UNIVERSITY OF ALBERTA

Synthesis and Analysis of Hetero-Divalent Oligosaccharides

by

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List of Abbreviations

4Å-MS	4Å Molecular sieves
Abs_{490}	Absorbance reading at 490 nm wavelength
Ac	Acetyl
AcO	Acetate
Ac_2O	Acetic Anhydride
anti-B	Monoclonal anti-B antibody
anti-Mouse	Anti-mouse IgM antibody
aq.	Aqueous
Asn	Asparagine
Bn	Benzyl
brine	Saturated aqueous sodium chloride
^tBu	tertiary-Butyl
Bz	Benzoyl
BzO	Benzoate
^{13}C-NMR	Carbon-13 nuclear magnetic resonance spectrum
Con A	Concanavalin A
CSA	10-Camphorsulphonic acid
CT	Cholera toxin
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCC	1,3-Dicyclohexylcarbodiimide
DCM	Dichloromethane

ΔG	Difference in Gibbs free energy
ΔH	Difference in enthalpy
ΔS	Difference in entropy
d	Doublet
d'	Secondary carbon; i.e.: bonded to two hydrogens
dd	Doublet of doublets
ddd	Doublet of doublet of doublets
dt	Doublet of triplets
DMAP	4-Dimethylaminopyridine
DMF	Dimethylformamide
ELISA	Enzyme-linked immunoassay
F_{ab}	Fragment: Antigen binding
F_c	Fragment: Crystallizing
GalNAc	NH-acetyl-D-galactosamine
GlcNAc	NH-acetyl-D-glucosamine
GTP	Guanidine triphosphate
HBG-B	Human blood group B antigenic determinant
^{1}H-NMR	Proton nuclear magnetic resonance spectrum
HMBC	Heteronuclear multiple bond correlation
HMQC	Heteronuclear quantum correlation
HOBT	1-Hydroxybenzotriazole
HRES-MS	High-resolution electrospray mass spectrometry
HRP	Horseradish peroxidase

Hz	Hertz
IC_{50}	Inhibition concentration at 50%
IgG	Immunoglobulin G
IgM	Immunoglobulin M
ITC	Isothermal calorimetry
J	Coupling constant value
J_d	Coupling constant value for doublet
J_t	Coupling constant value for triplet
Ka	Binding association constant
K_d	Binding dissociation constant
L	Linker
LRES-MS	Low-resolution electrospray mass spectrometry
LT	*Escherichia coli* heat-labile enterotoxin
Man	D-Mannose
MHz	Megahertz
m	Multiplet
NeuNAc	NH-acetylneuraminic acid
NHAc	NH-acetyl
NIS	*N*-iodosuccinimide
NMR	Nuclear magnetic resonance
NPhth	*N*-phthalimido
OBz	Benzoate
OPD	*o*-Phenylenediamine

PAMAM	Poly(amidoamine)
PBS	Phosphate buffered saline
Ph	Phenyl
$PhCH_3$	Toluene
PhSH	Thiophenol
ppm	Parts per million
Py.	Pyridine
quant.	Quantitative
q	Quartet
q'	Quarternary carbon; i.e.: bonded to three hydrogens
R_f	Retardation/retention factor
R.T./r.t.	Room temperature
s	Singlet
s'	Quarternary carbon; i.e.: bonded to no hydrogens
sat.	Saturated
Ser	Serine
t	Triplet
t'	Tertiary carbon; i.e.: bonded to one hydrogen only
TFA	Trifluoroacetic acid
TfOH	Trifluromethanesulphonic acid
Thr	Threonine
TLC	Thin-layer chromatography

CHAPTER 1

Introduction

1.1 Carbohydrates and Their Biological Functions

Carbohydrates are found in many processes serving many roles. Originally, carbohydrates were discovered to operate as vehicles for food storage or operating as structural motifs. Recent discoveries have revealed their roles in recognition, regulation of interactions with other molecules along with modifying the physical nature of the molecule they are attached to.[1, 2]

Recent developments regarding the many roles and functions of carbohydrates attached to lipids and proteins have created subclasses of biologically relevant molecules – glycoconjugates. These subclasses are referred to as glycoproteins, glycolipids (linked via inositol or sphingosine) and proteoglycans (proteins linked to high molecular weight oligosaccharides).[3]

Oligosaccharides can be linked to proteins via amide bonds at C_1 of the reducing-end sugar. In the case of *N*-linked-glycoproteins, the C_1 (of *N*-acetylglucosamine, GlcNAc) is bonded to the amide functional group of L-asparagine. *N*-linked-glycoproteins can be complex but always have the same core oligosaccharide fragment (Figure 1.1).[4] *O*-linked-glycoproteins feature C_1 (of *N*-acetylgalactosamine, GalNAc) bonded to the alcohol functional group of L-serine or L-threonine. Once again, structural diversity is based upon a set of oligosaccharides that are added by specific transferase enzymes (Figure 1.2).[2, 4]

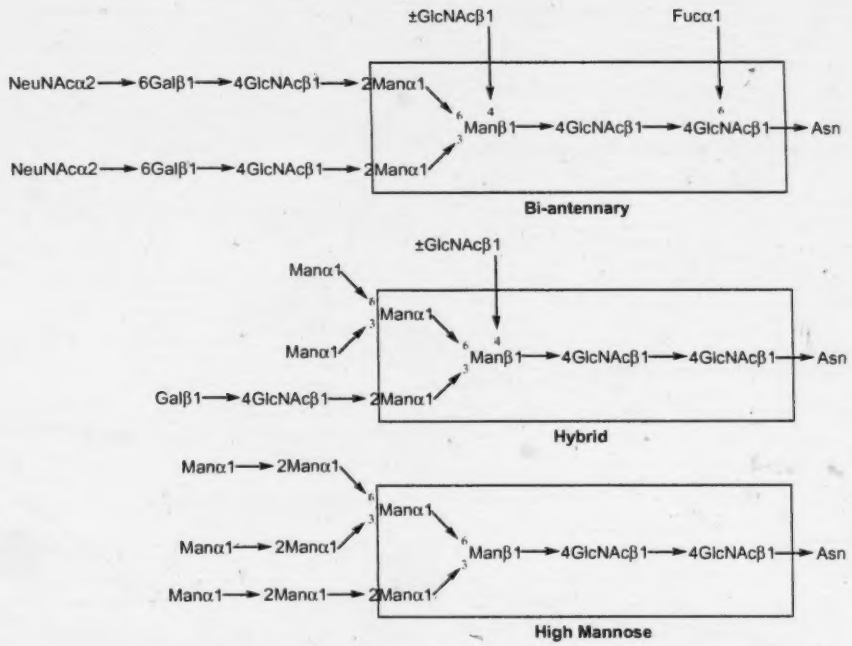


Figure 1.1: Examples of *N*-glycans[4]. the common "core pentasaccharide" is boxed-in.

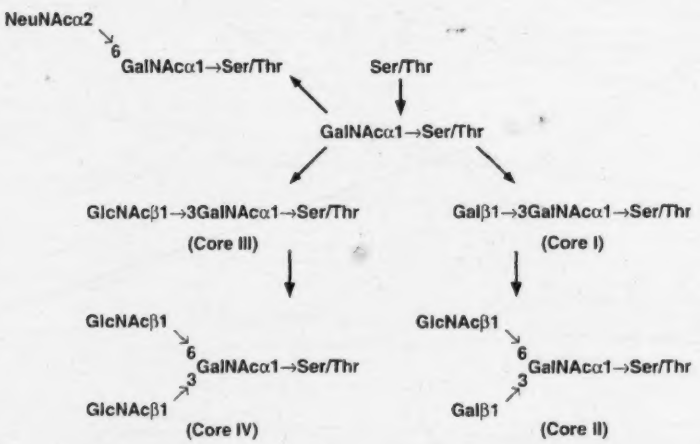


Figure 1.2: Biosynthesis of the core portions of O-glycans[4]

Glycolipids, a subclass of membrane lipids, are comprised of hydrophobic and hydrophilic moieties that are attached to a sphingosine framework. The hydrophobic portion(s) usually consists of long chain fatty acids, ranging from C_{14} to C_{24}, with a greater abundance of the hydrophobic portions consisting of palmitic acid (C_{16}) and oleic acid (C_{18}). When the free amine is protected with a long chain fatty acid, it is termed ceramide. In either framework, the hydrophilic moieties ranging from mono- to oligosaccharides, are attached via a β-glycosidic linkage to C_1 of the reducing sugar (Figure 1.3).[4, 5] Glycolipids are further divided into two categories based upon the presence or absence of *N*-acetylneuraminic acid as part of the oligosaccharide moiety. Gangliosides at neutral pH, are negatively charged due to the carboxylate functional group present on *N*-acetylneuraminic acid; glycosphingolipids are neutrally charged at pH 7.[4, 5]

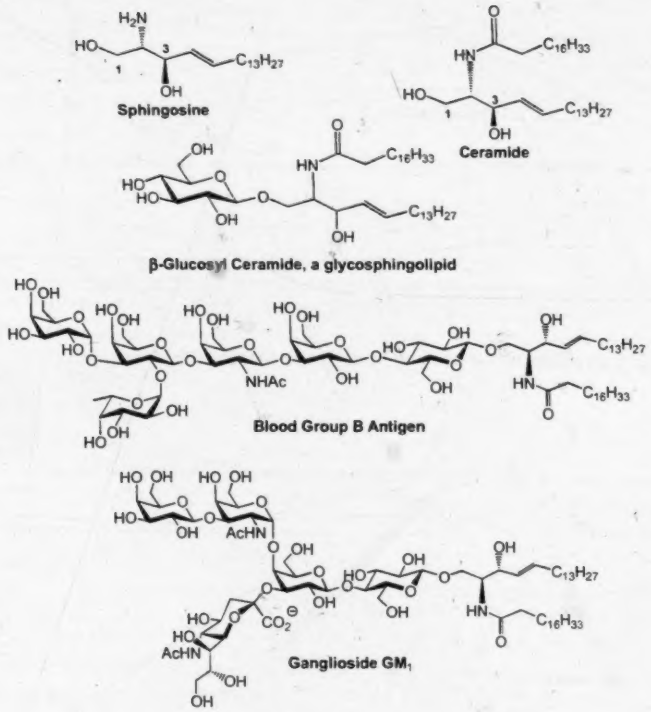


Figure 1.3: Examples of glycolipids

Glycoconjugates found on cellular surfaces are often used as receptors to facilitate tight binding.[6] These receptors facilitate normal biological processes such as immunology monitoring and control. However viruses, bacteria and fungi use these receptors to attach to and penetrate cell membranes leading to the spread of disease.[3, 6] The binding involves recognition of oligosaccharides on the cell membrane by proteins on the invading organism.

1.2 Multivalency

There are "a large number of biological processes [that] involve multivalent binding interactions, including cellular adhesion and signal transduction events"[7].

[8] The affinities of proteins binding to individual oligosaccharides are generally weak. The measured equilibrium dissociation constants range from mM to μM. This apparent "weakness" can be overcome by the use of multivalency wherein multiple proteins on a bacterium or virus bind multiple oligosaccharides at the same time. This "Glyco-Cluster Effect" can result in the amplification of carbohydrate signals in biological systems through the multivalent use of carbohydrates[9, 10]; it is an "affinity enhancement over and beyond what would be expected from the concentration increase of the determinant sugar in a multivalent ligand" (Figure 1.4).[11] This effect is demonstrated by multiple proteins, or multiple binding sites on the same protein binding to multiple oligosaccharides.[6, 12-14] The low individual dissociation constants of $10^{-3} - 10^{-4}$ M can be enhanced using multivalent approaches.[15, 16]

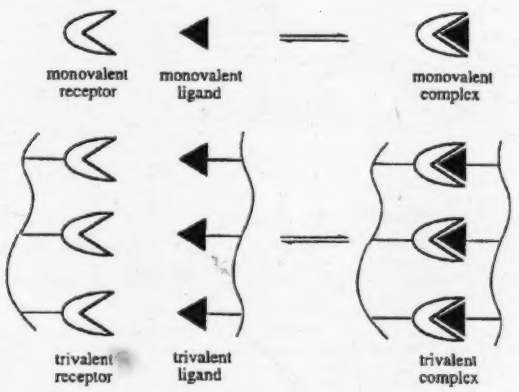


Figure 1.4: Schematic of mono- vs. multivalency[8]

Multivalent use of carbohydrates are prevalent in many natural systems: lectins (C-, I-, and P-type, galectins)[13], viruses (influenza, sendai, and rota),

bacteria (AB_5 toxins) and antibodies, to name a few.[6] Multivalent interactions have resulted in 2- and 3-dimensional structural frameworks between lectins and glycoconjugates.[7, 17, 18] Recently, chemists have begun to design many systems incorporating multivalency: Starfish[19], fingers[20], dendrimers[21, 22], polymers[8, 23, 24]

1.3 Objective and Hypothesis

Bacteria and toxins are known[6] to use oligosaccharides found on cellular surfaces in order to a) bind to the cell and b) release proteins into the intracellular cavity. The release of these proteins results in the destruction of the normal processes that occur in the cell often leading to the death of the cell. Nature uses polyvalent oligosaccharide presentations on cellular surfaces and multiple receptor sites effectively to achieve binding. The use of multiple oligosaccharide presentations and multiple receptor sites reduces the frequency of the bacterium and virus to become detached from the cellular surfaces since not all the oligosaccharides will be free at any given moment in time. Multivalency in fact amplifies each individual monovalent binding creating tighter binding which is reflected as a smaller dissociation constant (K_d).[12-16]

The objective of this thesis research is to use the Glyco-Cluster effect to examine whether a polyvalent presentation of weak-binding oligosaccharides will result in a tight binding of a polyvalent target protein; the oligosaccharides will be presented on a non-covalent template in solution. The resulting "templated multivalency" targeted is displayed in Figure 1.5. Template molecules will be

prepared in a hetero-divalent fashion. The ligand (Ligand for Multivalent Protein A in Figure 1.5) for the target multivalent protein (Multivalent Protein A) will be covalently attached through a linker (L) to an unrelated molecule. This latter molecule will be a ligand (Ligand for Multivalent Protein B) for a different multivalent protein (Multivalent Protein B). It is the objective of this thesis research that mixing the two proteins together in solution with the hetero-divalent ligand may result in tight-binding through templated multivalency (Figure 1.5).

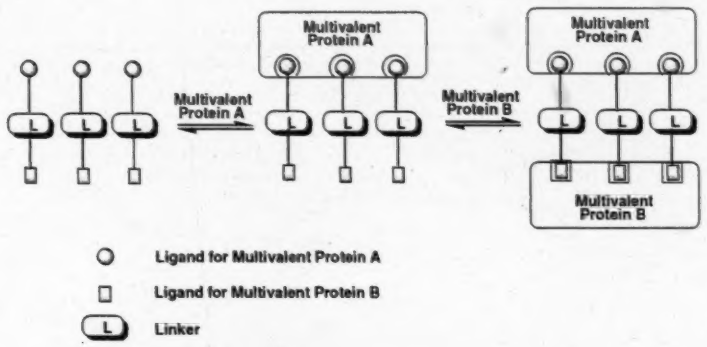


Figure 1.5: Proposed model for hetero-divalent binding

This project will examine the hypothesis using two different target proteins (Multivalent Protein A) and a common protein (Multivalent Protein B). Each of the target proteins' ligands (Ligand for Multivalent Protein A) to be synthesized should have low dissociation to their target proteins (discussion on the details of each protein to follow). The linker (L) must be water soluble as the proteins operate under physiological conditions and allow both Multivalent Proteins A and B to bind to their respective ligands while minimizing their steric interactions. This project will test the hypothesis that each of the ligands (Ligand A), when

presented hetero-divalently will: a) bind to their respective protein (Multivalent Protein A) in the presence of Multivalent Protein B bound to its ligand; b) have lower dissociation constants when compared to the ligands presented monovalently (Figure 1.5).

Based on these considerations it was decided to use three proteins: a monoclonal anti-Blood group B IgM antibody – § 1.7 as Multivalent Protein B and Cholera Toxin (CT) – § 1.4 and Concanavalin A (Con A) – § 1.6 as Multivalent Proteins A. The known ligands for these three unrelated proteins are the human blood group b trisaccharide (*O*-α-L-fucopyranosyl-(1→2)-[*O*-α-D-galactopyranosyl-(1→3)]-*O*-D-galactopyranoside (**1**), α-3,6-di-*O*-mannopyransoyl-mannoside (**2**) and lactoside (**3**) respectively (Figure 1.6). A short ethanolamine aglycon was to be used to attach all the ligands to the linker (L). The linker (L) to be used will be a modified polyethylene glycol chain. Different lengths of polyethylene glycols may have to be explored in order to achieve the highest affinity possible. The syntheses of the ligands for Con A, CT and anti-B antibody, and their coupling to two different lengths of PEG chains, will be presented in the next chapter. Before this, however, the biochemical properties of the proteins and the linkers themselves will be described in the remainder of the present chapter.

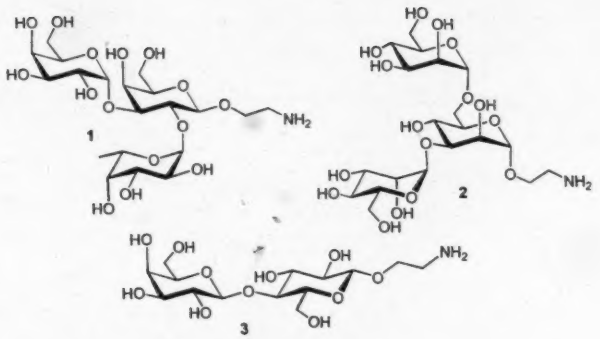


Figure 1.6: Proteins' ligands with novel aglycon

1.4 Cholera Toxin (CT), a pentavalent protein

The disease cholera is created by the pathogen *Vibrio cholerae*[25] via the release of the toxin. Cholera is known to cause diarrheal disease and dehydration[26]. The toxin is comprised of 6 subunits (1 A and 5 B subunits, Figure 1.7). The A subunit can be further divided into two parts (A and A_1). Worldwide deaths of over 10^6 people occur annually from the family of AB_5 toxins; it remains a severe medical problem.[27]

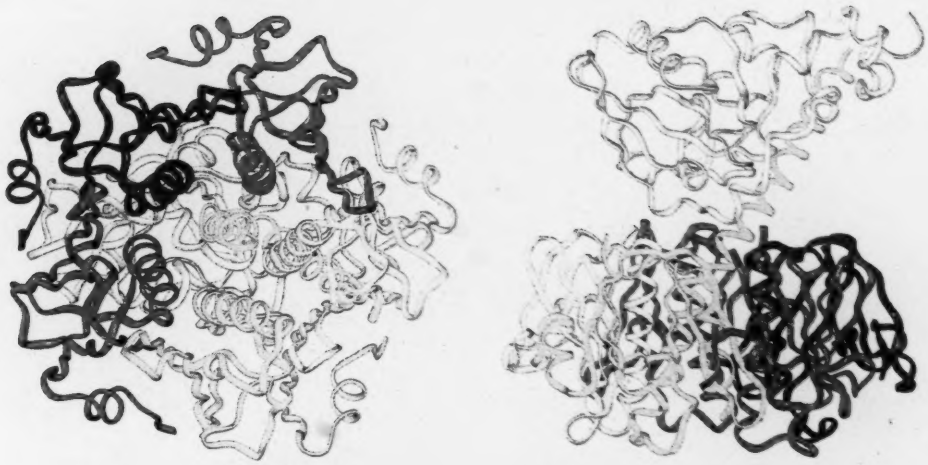


Figure 1.7: CT[28] a) dorsal view; b) lateral view (A subunits displayed in white and green; 5 B subunits displayed in red, blue, yellow, cyan and purple)

The toxin attaches to the outer membrane of the cell wall only if its natural ligand – Ganglioside GM_1 (**4**, Figure 1.9) – is present.[29, 30] Ganglioside GM_1 (**4**) is generally found on the cell walls of the small intestinal tract.[25, 26, 29-32] Studies have found that the toxin will only bind to the ligand via its B subunits. Individual B subunits will not bind to **4**[33], rather the pentameric form of B monomers affect the binding (K_d = 50 nM[34], Figures 1.8 and 1.9). Each monomer binds to the hydroxyl groups located on the terminal galactose (Ring E), *N*-acetylgalactosamine (Ring D), *N*-acetylneuraminic acid (Ring C) and the 2-OH of Ring B along with both *N*-acetyl groups on Rings C and D (Figure 1.9).[33]

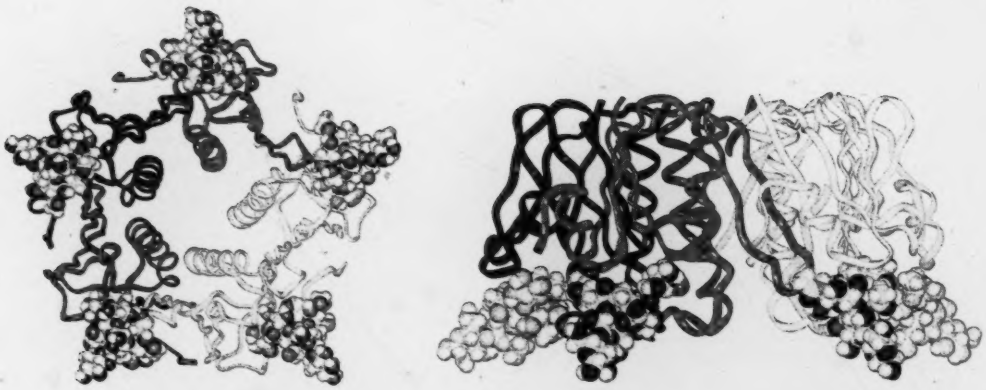


Figure 1.8: Pentameric B subunits of CT bound to ganglioside GM_1[28] –

a) dorsal view; b) lateral view

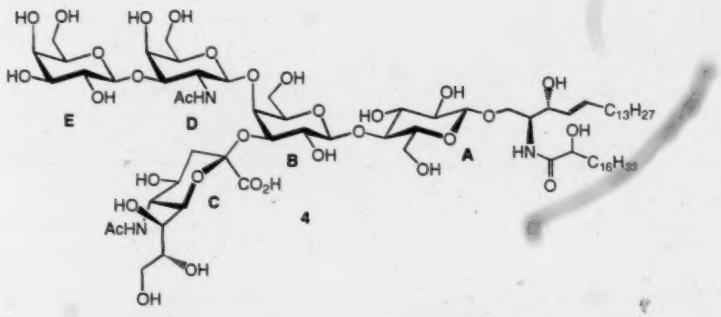


Figure 1.9: Structure of pentasaccharide ganglioside GM_1 (4)

Once bound, the toxin discharges its A_1 subunit through the B-pentamer cavity and through the cell membrane into the cell.[26, 27, 31, 33, 35, 36] Once secreted into the cell, the A_1 peptide catalyzes "ADP-ribosylation of residue Arg 187 in the α subunit of the trimeric protein G_s. The modified $G_{s\alpha}$ loses its GTPase activity and remains constitutively in its GTP-bound state, which in turn causes a continuous stimulation of adenylate cyclase. The resulting elevated levels of

cyclic AMP lead to massive loss of fluids, the characteristic pathology of enterotoxigenic disease."[27, 33, 36]

1.5 *Escherichia coli* Heat-Labile Enterotoxin (LT)

Cholera Toxin (CT) is part of a family of bacterial toxins. *Escherichia coli* Heat-Labile Enterotoxin (LT-I and II)[27, 33], Pertussis toxin, Diphtheria toxin, Shigella Toxin and *Pseudomonas aeuroginosa* exotoxin A are all related in structure and function to CT.[33] LT is known to cause traveler's diarrhea.[31] Specifically, LT-I has an 80% sequence homology in both the A and B subunits to CT (Figure 1.10).[26, 27] X-ray crystallography of LT[37] bound to lactose(**5**) revealed that the amino acids of the B monomer hydrogen bonded to galactose only.[37] A comparative analysis of the amino acids present in the binding sites of both CT and LT revealed that similar residues bound their respective ligands; arginine (LT) for histidine (CT) at position 13 and methionine (LT) for leucine (CT) at position 31 were the only exceptions.[27, 33, 37] Based upon this similarity, binding of **5** to CT was performed; the K_d was 18 mM.[25] Therefore lactoside **3** should be a good ligand for CT.

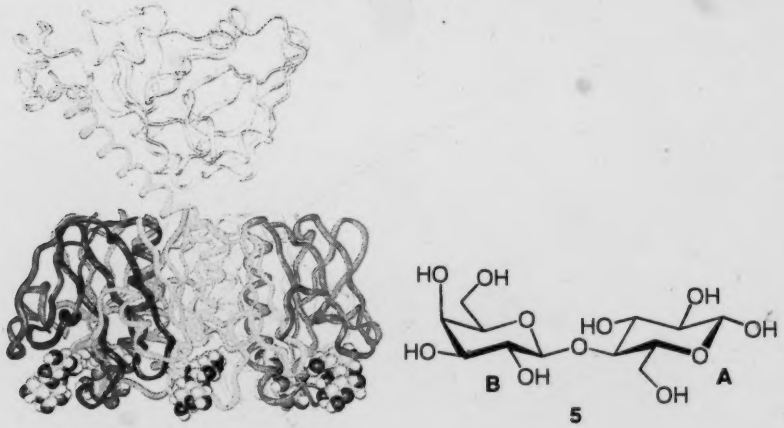


Figure 1.10: a) Lateral view of LT bound to 5[37]; b) lactose (5)

1.6 Concanavalin A (Con A), a multimeric protein

Lectins are proteins of non-immune origin that display affinity to carbohydrates without modifying them.[38, 39] Lectins were originally found in plants, but also occur in animal and invertebrates tissues.[38, 40] In many cases, the carbohydrate ligands are part of *N*-linked glycopeptides or glycolipids.[38, 40-42] As mentioned previously, the oligosaccharides of *N*-linked-glycoproteins are attached to L-asparagine.[4, 38, 43]

The lectin Con A (Figure 1.11) was the first lectin to be isolated[44, 45], sequenced[44, 46-48] and to have its three-dimensional structure determined by x-ray crystallography.[44, 46, 47, 49, 50] It was isolated from jack bean seeds (*Canavalia ensiformis*)[41, 45, 51] and has been classified as part of the glucose/mannose-specific lectins.[44, 51-53] Con A is the most extensively studied lectin[49]; the lectin is usually found in its dimeric or tetrameric forms based upon pH (dimers -- 6 < pH < 7 – tetramers).[46, 54] Each monomer consists of a sandwich of two β sheets; the

β sheets facilitate the monomers to dimerize (via hydrogen bonding) and to tetramerize (via salt links between lysine and glutamic acid residues and hydrogen-bonded solvent bridges between histidine and serine residues).[49, 55] Each monomer has one saccharide-binding site and two distinct metal-binding sites.[46, 47, 49, 50, 55] The metal-binding sites are acidic residues. This is consistent with the finding that the metals (mainly Ca^{2+} and Mn^{2+}) are released from the carboxylates under acidic conditions; binding of other metals to Con A has also been reported – Cd in lieu of Ca and Ni, Co, Zn and Cd as a substitute for Mn.[50, 54, 56-58] It is also important to note that the affinity of the monomers for saccharides is based upon the presence of the metals in their two binding sites.[44, 46, 49, 54]

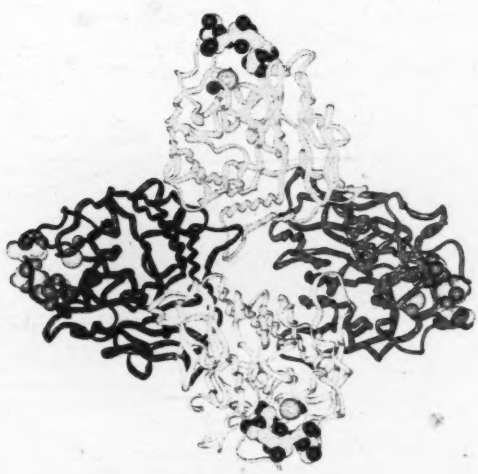


Figure 1.11: Crystal structure of Con A bound to trimannose[49]

Studies have shown that K_d for the binding of simple mono- and oligosaccharides to most lectins are between 10^{-3} to 10^{-6} M.[38, 41, 56-59] Con A has

the highest binding affinity for methyl-3,6-di-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside (Figure 1.12).[44, 52, 60] *N*-linked-glycoproteins have greater binding (as a result of extended site interactions) if the oligosaccharide has a trimannose fragment (Figure 1.13).[38, 41, 59] All three monosaccharide units are well recognized by Con A[49] through the use of hydrogen bonding and/or van der Waals contacts. The crystal structure of Con A – published by Bouckaert, et. al.[44] and Naismith et. al.[49] -- bound to methyl-3,6-di-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside (**6**) revealed that Ring F (α-(1→6)-Man*p*) resides in the monosaccharide binding site;[49] 3-, 4- and 6-hydroxyl groups are critical for tight binding.[44, 49, 52] While the reducing sugar's anomeric conformation is preferred to be α[49], interaction with hydroxyl groups of Rings D (reducing sugar) and E (α-(1→3)-Man*p*) creates an extended binding site.[52] The dissociation of the trisaccharide (K_d = 2.0 x 10^{-7} M)[58] from the lectin would occur less frequently when compared to methyl α-D-Man*p* (**7**, K_d = 1.2 x 10^{-4} M).[56, 58, 61]

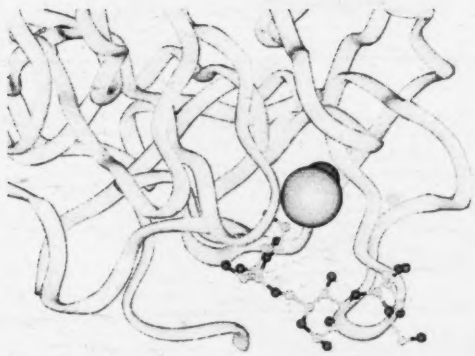


Figure 1.12: Enlargement of saccharide binding site[49]

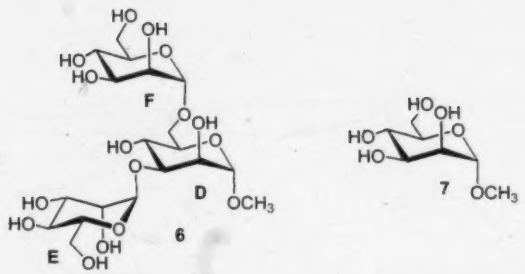


Figure 1.13: Methyl α-3,6-di-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside (6) and methyl α-D-mannopyranoside (7)

1.7 Anti-B IgM Antibody

"Antibodies to ABO antigen [oligosaccharide] structures are among the most predominant naturally occurring antibodies".[62] The roles of these antibodies are non-specific anti-bacterial defense along with general self-regulation of the immune system.[62-64] Antibodies are part of the human body's adaptive immunity;[65] they are the body's primary immune response.[66] They reside as monomers on the membranes of B cells located in the 10^9 lymphocytes found throughout the human body. Expression occurs when their corresponding antigens are exposed on cellular surfaces. Antigens are found on the surface of red blood [precursor][67-69] and other cells[68] (e.g.: digestive organ epithelial cells[67, 70]), and are important in cell development, cell differentiation and oncogensis.[69]

Each immunoglobulin monomer is the sum total of two heavy and two light chains.[62, 66] Both the heavy and light chains have variable and constant regions. In the overall Y structure of immunoglobulins, two heavy chains are linked together to form the stem of the Y structure known as the F_c (fragment that

crystallizes) region. Each of the two light chains coordinate with the remaining portion of the heavy chains forming the two arms of the Y structure known as the F_{ab} (fragment with antigen binding sites) (Figure 1.14). The uniqueness to each immunoglobulin (IgG vs. IgM) is a result of the different amino acid sequences found in the heavy chains. This difference attributes to the monovalency of IgG molecules compared to the pentavalency of IgM molecules.[65] Disulphide bonds between the IgM subunits along with bonding to a J-chain facilitate the soluble secretion of these antibodies as a pentamer.[66, 71-73]

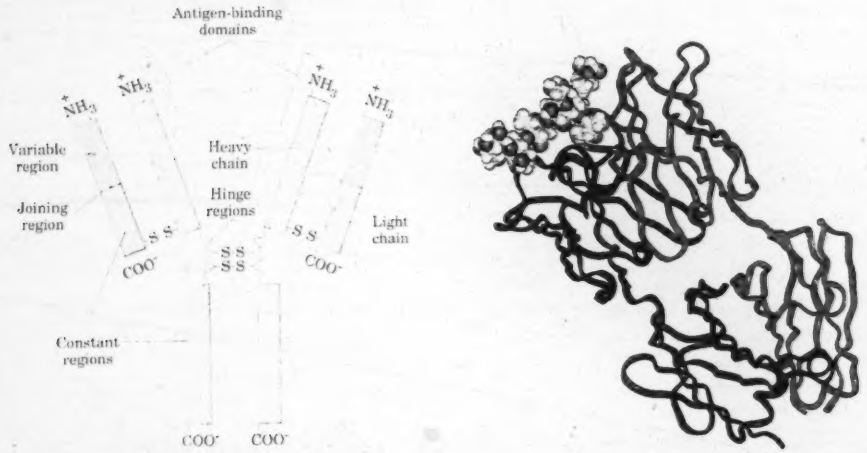


Figure 1.14: a) Schematic of antibody heavy and light chains[5]; b) F_{ab} region of antibody bound to oligosaccharide[74]

Though immunoglobulin proteins have low affinities, their polyvalency provides them with a strong ability to bind to their antigens.[66] Examination of the Y structure of IgG displays two F_{ab} sites; the pentavalent arrangement of IgM provides 10 binding sites.[75] It is important to note that each immunoglobulin has its own antigen. From the many epitopes that immunoglobulin G and M proteins bind, there are a set of oligosaccharides protruding at the non-reducing termini of

N- and *O*-glycoproteins and glycolipids that are recognized as antigens.[62, 66, 76-78] The recognition by immunoglobulins is dependent upon the expression of these antigens. IgM anti-A and anti-B antibodies will search for antigens A and B respectively (Figure 1.15) when released from the lymphocytes. It is important to note that individuals that produce A antigens cannot produce anti-A antibodies. All glycoconjugates that contain the A antigen are removed from the human body of individuals that have anti-A antibodies.[62, 68, 69, 79]

Each of these antigens is characterized uniquely via the presence of an individual saccharide. Anti-A antibody will bind to the trisaccharide that bears a terminal *N*-acetylgalactosamine (GalNAc) while anti-B antibody requires the presence of a terminal galactose.[68] Within the class of IgG proteins, there are antibodies (anti-Gal B, anti-Gal A/B and anti-Gal) specific to antigens bearing the terminal α-galactosyl fragment found on the non-reducing end of glycoconjugates.[76, 80] The IgG anti-Gal B antibody is found in blood type A and O individuals while the anti-Gal A/B antibody is expressed only in individuals with blood type O. Anti-Gal B recognizes the Galα(1→3)Gal portion of the B antigen (**7**). Anti-Gal A/B recognizes both A and B antigens.[76] Anti-Gal will not recognize the Galα(1→3)Gal portion of A (**8**) and B (**7**) antigens due to the presence of α(1→2)Fuc (Figure 1.15). Foreign bacteria and viruses containing exclusively the Galα(1→3)Gal epitopes (**9**), anti-Gal antibody will remove them from the human body.

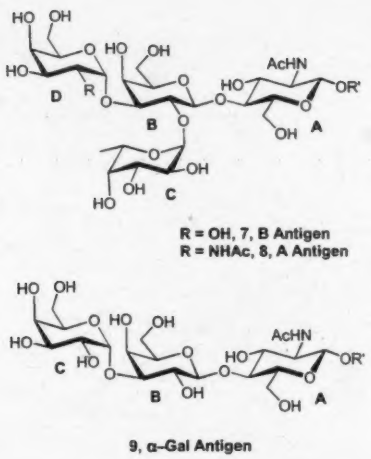


Figure 1.15: Structures of AB and α-Gal antigens[67, 76, 81]

1.8 Dendrimers: a class of covalent polyvalent ligands

The use of dendrimers has become prevalent in the field of supramolecular chemistry. They are used in biomimetic catalysis, building blocks, molecular carriers and as drug delivery vehicles.[82-88] This intrinsic nature of dendrimers is superior to other three-dimensional supramolecular structures such as micelles and liposomes which are based upon the aggregation of smaller components; the aggregation is based upon dynamic equilibrium or thermodynamic factors.[89-92]

Dendrimers are made up of dendrons – branched, wedge-like structures – emerging from a multivalent center[89]. The overall globular polymer structure is composed, either divergently or convergently[83], from a synthetically designed central core and dendrons that are bonded covalently. The control in the synthetic design facilitates the effective optimization of the overall size, shape,

chemical property(ies) or characteristics.[89, 93] Drug carriers involving dendrimers can be designed with a low polydispersity index (≤ 1.1); uniform molecular weight distribution between batches is essential for the measurement of pharmacokinetic behaviour.[89] The first examples were the generation of poly(amidoamine) (PAMAM) dendrimers (Figure 1.16). However, their medicinal properties were not favourable. Placing units such as (trishydroxymethyl) amino methane at the periphery improved their properties thereby facilitating their use as drug delivery vehicles. Recently, scientific groups have modified the basic framework of dendrimers attempting to make syntheses consistent and simple while maintaining their favourable medicinal properties. Frechet[89] et al. have displayed the use of 2,2-bis(hydroxymethyl)propanoic acid as scaffolds for dendrimer formation while Stoddart[94] et. al. have used α-mannopyranose units at the periphery of PAMAM based dendrimers in order to observe lectin (Con A) ligand interactions (Figure 1.16).

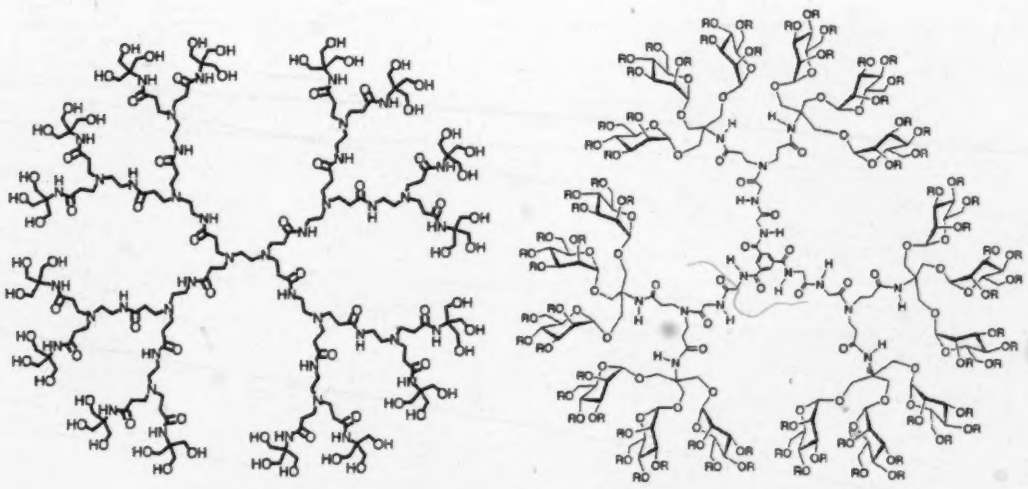


Figure 1.16: Examples of dendrimeric systems[94, 95]

1.9 Polyethylene Glycols: water soluble linkers

Polyethylene glycols are synthesized from the aqueous anionic polymerization of ethylene oxide and is comprised of either linear or branched polyether chains that are terminated with hydroxyl groups.[96, 97] The polymer has been characterized for its water soluble and biocompatible properties while featuring non-toxic and non-immunogenic behaviour; Similar to dendrimers, the development of the polymerization techniques feature polyethylene glycols with a low polydispersity index value (< 1.05).[97-99]

The favourable biocompatibility has resulted in a surge in their use as carriers of proteins, oligosaccharides and other organic molecules (e.g.: Taxol and Folic Acid attached to a Carboplatin frame via polyethylene glycol, Figure 1.17) via solution and solid-phases.[97, 100] High molecular weight polyethylene glycols have been used as carriers for low molecular weight drugs[98, 99]. This allows for better distribution and longer blood circulation period.[98, 101] Polyethylene glycol carriers attached to drugs have benefited from lowered immunogenecity and produced better tumour uptake.[98, 102-104] Similar to dendrimers, polyethylene glycols may be used as drug delivery vehicles facilitating better pharmacological and biological properties.[96] Late phase clinical trials of drugs on a polyethylene glycol framework (e.g. Pegademase – adenosine deaminase on Adagen, pegaspargase – L-asparaginase on Oncaspar, PegInteferon α2b – INT-α2b on PegIntron) have displayed the use of polyethylene glycol as an effective pharamaceutical and medicinal tool.[96, 105]

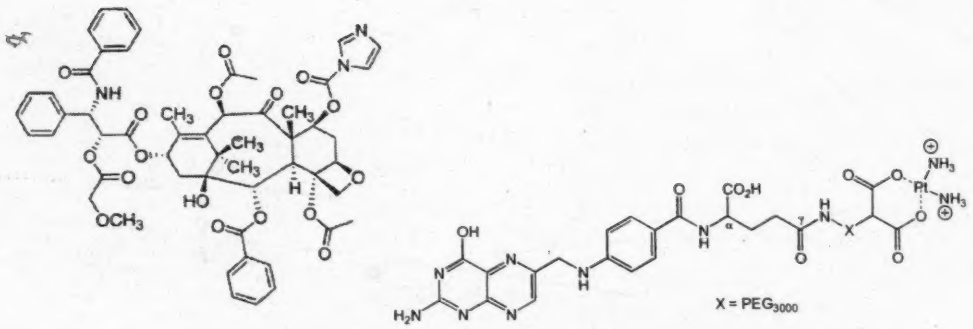


Figure 1.17: Examples of drugs built involving polyethylene glycol[97, 98, 100]

CHAPTER 2

Synthesis of Hetero-Divalent Oligosaccharide Ligands

2.1 Retrosynthetic Analysis of Hetero-Divalent Oligosaccharide Ligands

The manufacture of all four hetero-divalent ligands (**10**, **11**, **12** and **13**) required a synthetic plan that facilitated coupling of the synthesized ligands (**1**, **2** and **3**) and minimal number of modifications post-coupling. When examining both sets of hetero-divalent ligands (Figure 2.1) each pair can be sub-divided into four building blocks: two ligands (**1** and **2**, or **1** and **3**) and the pair of linkers (**14** and **15**). The homogeneity in both sets of hetero-divalent ligands is the presence of the human blood group B antigenic determinant (HBG-B, **1**) and the linkers (**14** and **15**). To synthesize all four hetero-divalent ligands (**10**, **11**, **12** and **13**), it would be easiest to attach the Ligand for Con A (**2**) and the Ligand for CT (**3**) to products that already incorporate **1** bound to the linkers (Figure 2.1). The ligand **1** may be coupled to each of the two linkers (**14** and **15**) once they are prepared. The ligands **2** and **3** could then be coupled to both ligand **1** bound to the short linker (**16**) and ligand **1** bound to the long linker (**17**). This would result in the formation of the desired four hetero-divalent ligands (**10**, **11**, **12** and **13**). The synthetic schemes for each of the individual ligands and linkers are described in detail below.

As displayed in Figure 2.1, amide coupling reactions would be used in order to bind all the pieces together. The faster rate of reactivity to form amides

over esters allows for the presence of deprotected sugars. The use of deprotected oligosaccharides helps remove the burden of having to deprotect the final four hetero-divalent ligands.

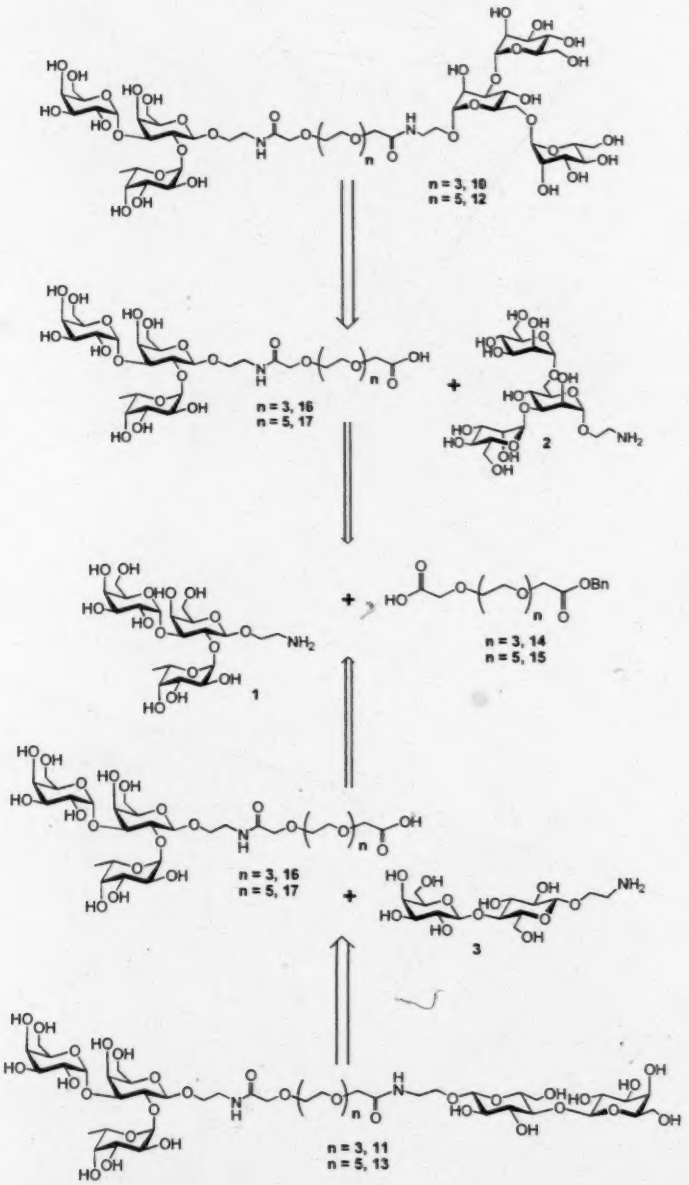


Figure 2.1: Retrosynthetic analysis of hetero-divalent ligands

2.2 Synthesis of the HBG-B (**1**)

The target molecule **1** was synthesized according to the retrosynthetic analysis (Figure 2.2) developed from the previous synthetic efforts of Lemieux and Sinaÿ.[106-108] The protected trisaccharide (**18**) was synthesized using a convergent approach using the monosaccharides **19**, **20** and **21**. The synthesis was based upon the sequential glycosylations of **20** and **21** at the 2- followed by the 3-position of **19**.[106-108] Both **19** and **21** are derived from D-galactopyranose (**22**); **20** is easily derived from L-fucopyranose (**23**, 6-deoxy-L-galactopyranose).

In both sets of glycosylations, it is important to note that the choice of benzyl protecting groups at the 2-position of both sets of donors (**20** and **21**) is purposeful. The leaving group in both of the donors is a thio-derivative. The 1,2-trans relationship facilitates a carbonyl based protecting group (e.g.: acetate, benzoate, phthalimido) to participate in the stereoselectivity of the glycosylation reaction.[109] Benzyl protecting groups at the 2-position have no carbonyl functionality; they cannot participate, resulting in the formation of the kinetically as well as the thermodynamically favoured α-anomer.

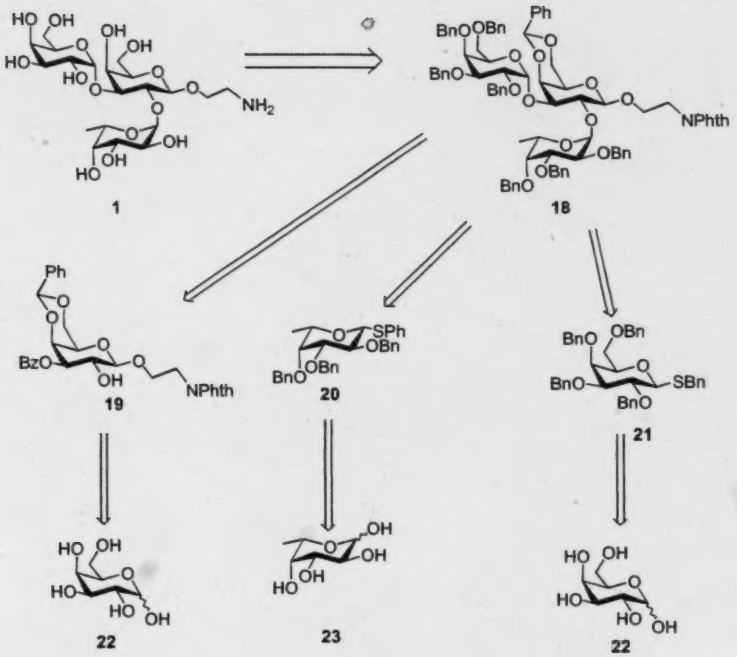
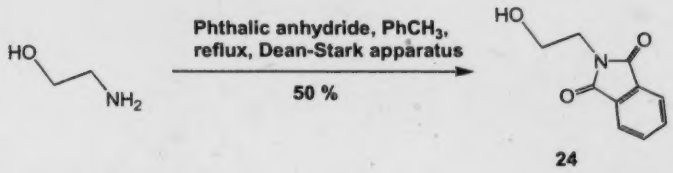


Figure 2.2: Retrosynthetic analysis of the HBG-B (1)

2.2.1 Synthesis of *N*-phthalimidoethanol (**24**)

The reaction of ethanolamine with phthalic anhydride was originally attempted using chloroform as the solvent[110, 111]; however, the reaction failed. A second effort at protection of the free amine was successful by refluxing in toluene. A Dean-Stark apparatus was used to remove the by-product H_2O from the reaction. Crystallization from chloroform afforded the desired product **24** in 50% yield (see Scheme 2.1). This aglycon was used in the syntheses of all three ligands. The ligands would then be bonded to the linker using standard amide coupling conditions.



24

Scheme 2.1: Synthesis of *N*-phthalimidoethanol (24)

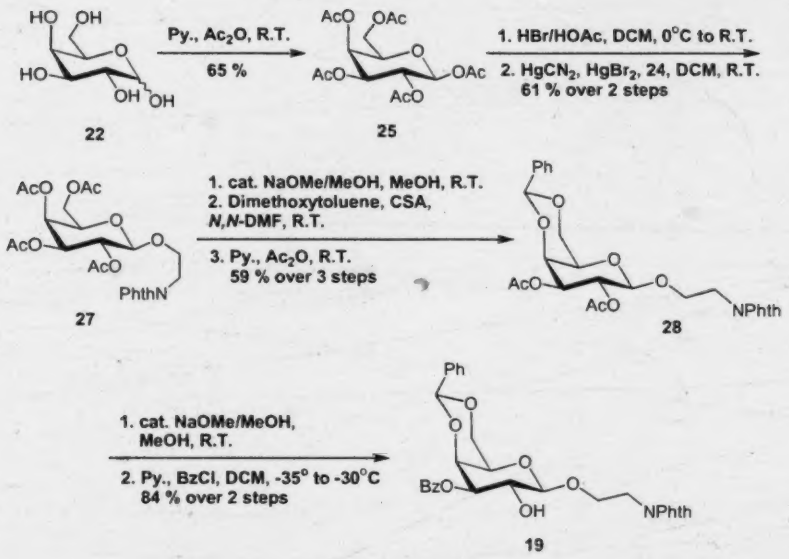
2.2.2 Synthesis of 2-phthalimidoethyl 3-*O*-benzoyl-4,6-*O*-benzylidene-β-D-galactopyranoside (**19**)

The first step in the synthesis of **19** was the acetylation of D-galactopyranose. A large scale (50 g, 0.28 mol) reaction afforded 70.7 g (65% yield) of **25**. Bromination of **25** using HBr/HOAc in DCM produced the desired α-anomeric bromide (**26**). Though there was a downfield shift of H_1 from 5.68 ppm to 6.70 ppm, the coupling constant (J = 4.0 Hz) did not change. Immediate slow addition of the α-bromo anomer (dissolved in DCM) in a glycosylation reaction with **24** in the presence of $HgBr_2$ and $HgCN_2$[109, 112, 113] in DCM at room temperature produced **27** as the β-anomer in 61% yield over 2 steps. A drastic upfield shift of H_1 (4.43 ppm, J = 7.8 Hz) confirmed the presence of the β-glycoside.

The tetra-*O*-acetate **27** was de-acetylated using Zemplén conditions – catalytic NaOMe in MeOH at room temperature – and this was followed by regioselective benzylidene protection of the 4,6-diol. The remaining 2,3-diol was acetylated using pyridine and acetic anhydride in order to facilitate isolation of the

product. The desired product **28** was isolated after chromatography in 59% yield over 3 steps (Scheme 2.2). The loss of acetyls resulted in the upfield shifts of H_4 and both H_6 protons observed in the ^{1}H-NMR spectrum. The final two steps involved Zemplén deacetylation of the 2,3-di-*O*-acetate followed by selective benzoylation using benzoyl chloride. Initially, selective benzoylation was attempted using benzoyl chloride and imidazole with 1,2-dichloroethane as solvent.[107] However, the procedure called for the reaction to operate at reflux temperatures; this facilitated the migration of the desired 3-*O*-benzoyl to the neighbouring free alcohol. Isolation of the 3-*O*-benzoyl derivative and its undesired regioisomer created the need to find a much milder procedure.

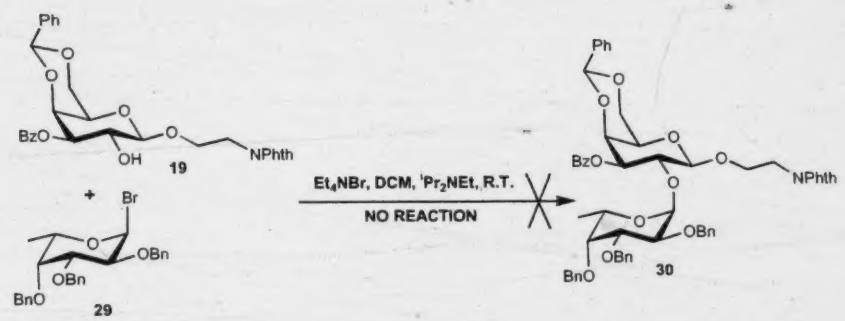
The regioselective protection of the 3-hydroxy group was accomplished by using pyridine instead of imidazole. The reactivity of pyridine allowed the reaction to occur at a significantly lower temperature range (-35°C to -30°C).[109, 110, 112, 114] Analysis confirmed the isolation of the desired 3-*O*-benzoyl product (**19**) exclusively in 84% yield over 2 steps. Examination of the ^{1}H-NMR spectra of **28** and **19** revealed that the H_3 remained downfield due to the electron-withdrawing nature of the acetate and benzoate groups. The HMBC spectrum revealed the unique $^3J_{CH}$ between H_3 and $\underline{C}$=O of the 3-*O*-benzoate. Along with mass spectrometry confirmation, there was sufficient evidence that the synthesis of **19** had been successfully completed.



Scheme 2.2: Synthesis of 2-phthalimidoethyl 3-*O*-benzoyl-4,6-*O*-benzylidene-β-D-galactopyranoside (19)

2.2.3 Synthesis of phenyl 2,3,4-tri-*O*-benzyl-1-thio-β-L-fucopyranoside (**20**)

Once synthesized, the initial attempt to add 2,3,4-*O*-benzylated fucose to the 2-position of **19** involved the use of fucosyl bromide (**29**) as the donor[106] (Scheme 2.3). However, the glycosylation reaction failed; a variety of products were obtained from which the desired product (**30**) could not be isolated in appreciable yield.

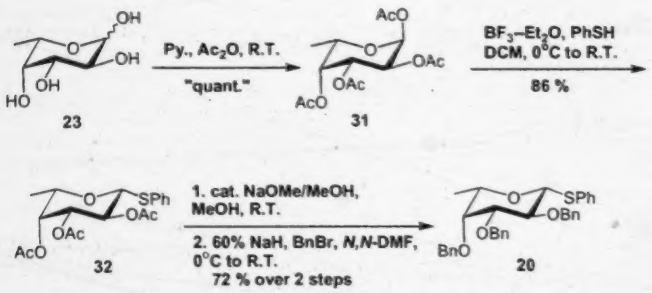


Scheme 2.3: Failed glycosylation using fucosyl bromide

A second attempt at the glycosylation was made using a thiophenyl donor (Scheme 2.4). The synthesis of the thiophenyl donor (**20**) began with the acetylation of L-fucopyranose using pyridine and acetic anhydride; a quantitative yield was obtained. The anomeric acetate (**31**) was converted into the thiophenyl donor using thiophenol and boron trifluoride-diethyl ethereate[115]; the reaction flask was allowed to warm to room temperature from 0°C after the additions were completed. Isolation of the exclusive β-anomer **32** was achieved in an 86% yield (Scheme 2.4). Analysis using ^{1}H-NMR spectroscopy and mass spectrometry clearly revealed the replacement of the anomeric *O*-acetate group with the thiophenyl group. The ^{1}H-NMR spectrum displayed the migration of H_1 upfield from 6.71 to 4.52 ppm; the anomeric configuration had also changed. The 1,2-cis relationship between H_1 and H_2 (J = 3.6 Hz) had become trans (J = 10.2 Hz); therefore the anomeric orientation had switched from α to β .

The tri-*O*-acetate (**32**) was de-acetylated using Zemplén conditions at room temperature. The resulting 2,3,4-triol was benzylated using NaH and BnBr in DMF; both of the additions of NaH and BnBr to the reaction flask were

performed at 0°C followed by stirring at room temperature; the tri-*O*-benzylated donor **20** was isolated in 72% yield over 2 steps. Analysis clearly confirmed the replacement of all three *O*-acetates with benzyl groups. There were upfield migrations of H_2, H_3 and H_4 along with large correlations between the respective benzylic carbons and H_2, H_3 and H_4. Correlations ($^3J_{CH}$) observed in the HMBC spectrum between the benzylic carbons and H_2, H_3 and H_4 solidified the analysis. Mass spectrometry revealed that there were no traces of unreacted benzyl bromide.

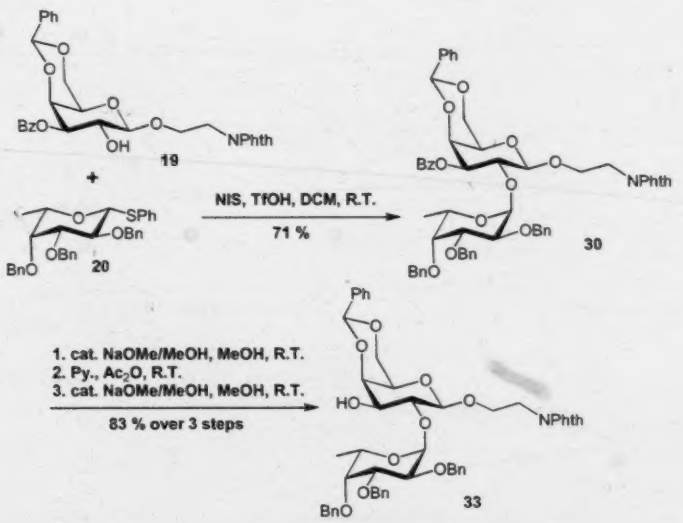


Scheme 2.4: Synthesis of phenyl 2,3,4-tri-*O*-benzyl-1-thio-β-L-fucopyranoside (20)

2.2.4 Synthesis of 2-aminoethyl α-L-fucopyranosyl-(1→2)-[α-D-galactopyranosyl-(1→3)]-β-D-galactopyranoside (**1**)

The glycosylation involving **19** and **20** was performed using *N*-iodosuccinimide and trifluoromethanesulphonic acid at room temperature.[116, 117] A disaccharide was isolated in 71% yield. Examination of the ^{1}H-NMR spectrum revealed a new signal ($H_{1'}$, 5.39 ppm) with a coupling constant (J = 3.6 Hz)

consistent with a 1,2-cis relationship between $H_{1'}$ and $H_{2'}$ (Scheme 2.5). The bonding of the 2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl group to the 2-position of **19** resulted in a greater shielding of H_2; the signal had migrated upfield (4.34 to 4.03 ppm). Analysis of the HMBC spectrum displayed $^3J_{CH}$ between the $C_{1'}$ and H_2 and C_2 and $H_{1'}$. The HRES-MS confirmed that the molecule had the desired molecular weight. Therefore, the glycosylation produced the disaccharide with the correct α(1→2)-bond between the tri-*O*-benzylated-L-fucopyranose and the 2-position of **19**.

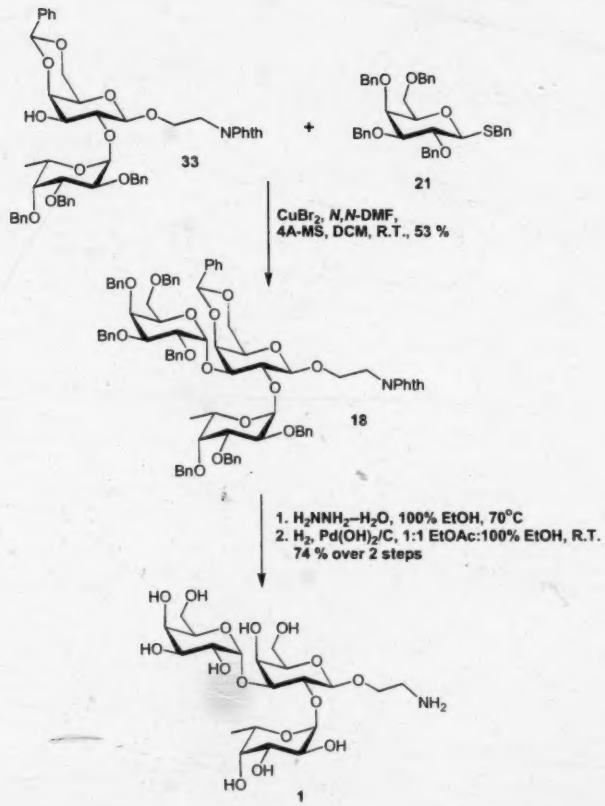


Scheme 2.5: Synthesis of 2-phthalimidoethyl 2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl-(1→2)-4,6-*O*-benzylidene-β-D-galactopyranoside (33)

Subsquent deprotection of the 3-*O*-benzoyl group from **30** was made cumbersome due to phthalimido ring opening during purification. Re-acetylation of the 3-alcohol using pyridine and acetic anhydride resulted in phthalimido ring

closure. Zemplén de-acetylation of the 3-*O*-acetate produced the desired acceptor **33** in 83% yield over 3 steps (see Scheme 2.5).

The final glycosylation involved the donor **21** and acceptor **33**. The donor was not synthesized in the Hindsgaul lab, however, it was kindly donated to the Hindsgaul lab by Synsorb Biotech Inc. The glycosylation was performed by adding the donor **21** to the reaction flask containing $CuBr_2$, DMF and **33** stirred in DCM in the presence of powdered 4Å-MS at room temperature.[118] A trisaccharide was isolated in 53% yield (Scheme 2.6). Once again, the ^{1}H-NMR spectrum revealed the presence of a new signal ($H_{1''}$, 5.45 ppm) with the coupling constant (J = 3.6 Hz) consistent with a 1,2-cis relationship between $H_{1''}$ and $H_{2''}$; surprisingly, the H_3 signal had shifted downfield (3.59 to 4.02 ppm). Examination of the HMBC spectrum revealed two $^3J_{CH}$ between $C_{1''}$ and H_3 and C_3 and $H_{1''}$. Confirmation of the desired molecular weight by HRES-MS solidified the conclusion that the glycosylation reaction produced the desired $\alpha(1\rightarrow3)$-bonded trisaccharide **18**. Deprotection using hydrazine-hydrate in anhydrous ethanol[111, 119] under reflux followed by hydrogenolysis using Pearlman's catalyst [$Pd(OH)_2$/C] produced the completely deprotected trisaccharide **1** in 74% yield over 2 steps. HRES-MS confirmed that a compound with the desired molecular weight had been synthesized and the ^{1}H-NMR spectrum illustrated that all three H_1 signals had been conserved (H_1, 4.63 ppm, 8.0 Hz and $H_{1'}$/$H_{1''}$, 5.24 ppm, both 3.5 Hz). The trisaccharide synthesized was the desired HBG-B (**1**).



Scheme 2.6: Synthesis of 2-aminoethyl α-L-fucopyranosyl-(1→2)-[α-D-galactopyranosyl-(1→3)]-β-D-galactopyranoside (1)

2.3 Synthesis of the Ligand for Con A (**2**)

The retro-synthetic analysis of **2** (Figure 2.3) was developed from the synthetic efforts of Furneaux, Ogawa, Roy and Krepinsky.[113, 120-122] Once again, the protected trisaccharide (**34**) could be easily constructed by the consecutive addition of 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl bromide **35** at the 3- and 6-positions of 2-phthalimidoethyl 2-*O*-benzoyl-4,6-*O*-benzylidene-α-D-mannopyranoside (**36**). The 1,2-trans relationship between the 1-Br and 2-OAc

in **35** will dictate the stereoselectivity in both sets of glycosylations. The axial orientation of the 2-OAc would allow the carbonyl oxygen to donate one of its lone pairs to stabilize the developing carbocation as the bromide is being abstracted.[109, 113] This neighbouring group participation also facilitates the stereoselective nucleophilic attack from the bottom face of the molecule thus creating the desired α-glycoside. Both **35** and **36** could be synthesized from D-mannopyranose (**37**).

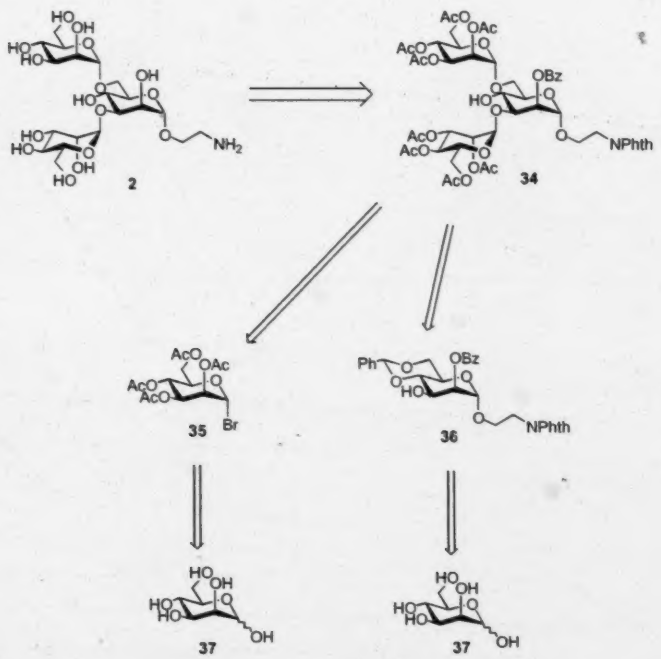


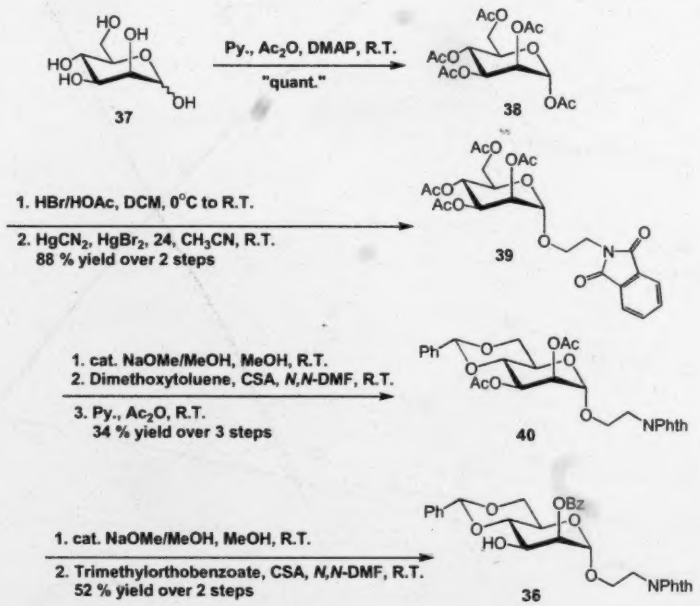
Figure 2.3: Retrosynthetic analysis of ligand for Con A (2)

2.3.1 Synthesis of 2-phthalimidoethyl 2-*O*-benzoyl-4,6-*O*-benzylidene-α-D-mannopyranoside (**36**)

Acetylation of D-mannopyranose with pyridine, acetic anhydride and 4-dimethylaminopyridine as the catalyst at room temperature over a period of 15 hours gave the α-anomer of **38** in a quantitative yield. NMR analysis displayed the presence of 5 $COC\underline{H}_3$ peaks. The orientation of the H_1 was determined to be equatiorial (6.06 ppm, J = 2.0 Hz). Bromination of **38** using HBr/HOAc in DCM at room temperature furnished the α-bromo product. The downfield shift of H_1 to 6.27 ppm while maintaining the small coupling constant (J = 1.2 Hz) consistent with a 1,2-di-equatorial relationship between H_1 and H_2, confirmed the formation of the α-bromo donor **35**. The mannopyranosyl bromide **35** donor was immediately reacted with **24** using Helferich conditions ($HgBr_2$, $HgCN_2$)[109, 112, 113] in dry CH_3CN at room temperature yielding **39** in 88% over 2 steps (Scheme 2.7). The ^{1}H-NMR spectrum displayed an upfield shift of H_1 (5.42 to 4.69 ppm). The thermodynamically favoured α-glycoside was formed.

The use of Zemplén de-acetylation conditions at room temperature gave the tetra-ol. Dimethoxytoluene under acidic conditions was used in order to regioselectively protect the 4,6-diol. However, the reaction often produced the di-benzylidenated product. Compared to the trans nature in galactopyranose, the cis-orientation of the 2,3-diol in mannopyranose created a low activation energy barrier thereby facilitating the formation of the bis-acetal alongside the desired 4,6-*O*-benzylidene product. Several batches of the regioselective protection

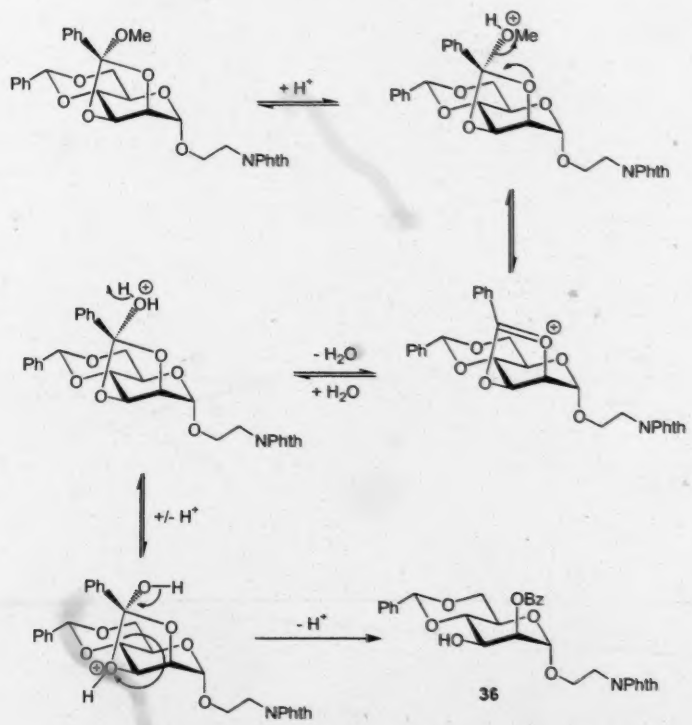
produced enough of the desired 4,6-*O*-benzylidenated product in order to proceed further. The 2,3-cis-diol was acetylated using pyridine and acetic anhydride at room temperature; the desired 2,3-*O*-acetate-4,6-*O*-benzylidenated product **40** was isolated after purification in 34% yield over 3 steps (Scheme 2.7). Comparison of the ^{1}H-NMR spectra of **39** and **40** showed that there was a net loss of 2 acetates. Upfield shifts of H_4 (5.62 to 4.15 ppm) and both sets of H_6 signals (4.30 and 4.19 to 4.09 and 3.57 ppm) confirmed that the protection of the 4,6-diol had occurred successfully.



Scheme 2.7: Synthesis of 2-phthalimidoethyl 2-*O*-benzoyl-4,6-*O*-benzylidene-α-D-mannopyranoside (40)

The final modification called for the regioselective benzoyl protection of OH-2. This was performed by first deprotecting the 2,3-*O*-di-acetate **40** using

Zemplén conditions at room temperature. The use of trimethylorthobenzoate under acidic conditions[123] furnished the desired 2-*O*-benzoate **36** in 52% yield over 2 steps (Scheme 2.7). The collapse of the 2,3-intermediate orthoester had occurred during chromatography. The acidic nature of silica gel resulted in the protonation of the methoxide facilitating its loss. Nucleophilic attack at the carbocation by H_2O followed by proton abstraction from the hydroxonium ion completed the hydrolysis. Collapse of the hydroxy acetal occurred in a kinetically driven axial fashion providing the 2-*O*-benzoate product (Scheme 2.8).[109, 112] On balance, H_3 had shifted upfield (5.73 to 4.29 ppm) while H_2 remained downfield (5.67 to 5.62 ppm).

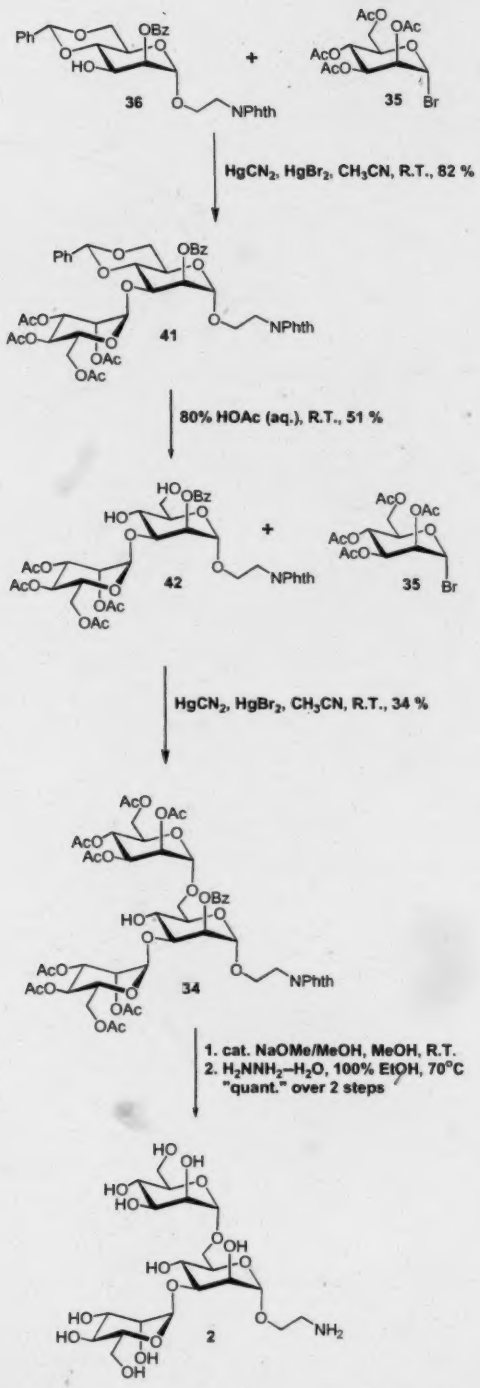


Scheme 2.8: Mechanism for 2-*O*-benzoate protection

2.3.2 Synthesis of 2-aminoethyl α-D-mannopyranosyl-(1→3)-[α-D-mannopyranosyl-(1→6)]-α-D-mannopyranoside (**2**)

The stereoselective glycosylation using **35** and **36** was performed using Helferich conditions.[109, 112, 113] After isolation, the donor (prepared from conversion of the anomeric acetate **38** to anomeric bromide **35**) was immediately dissolved in dry CH_3CN then added to the reaction flask containing the acceptor **36**, $HgBr_2$ and $HgCN_2$ in CH_3CN. After stirring at room temperature for 2.5 h, purification of the reaction mixture led to the isolation of a disaccharide in 82% yield over 2 steps. The ^{1}H-NMR spectrum displayed a new signal ($H_{1'}$, 5.46 ppm) with a coupling constant (J = 1.8 Hz) consistent with a 1,2-di-equatorial relationship between $H_{1'}$ and $H_{2'}$; the H_3 signal had shifted slightly downfield (4.29 to 4.55 ppm). The HMBC spectrum revealed the presence of two $^3J_{CH}$ between C_3 and $H_{1'}$ and C_1 and $H_{3'}$. Verification of the molecular weight by HRES-MS solidified the conclusion that the desired α(1→3) disaccharide **41** had been synthesized (Scheme 2.9).

The disaccharide was then subjected to benzylidene acetal hydrolysis using 80% HOAc (aq.) stirring for 4 h. Removal of the 4,6-*O*-benzylidene ring was achieved in 51% yield. The indistinct shifting of H_4 (4.13 to 4.07-4.15 ppm) and both sets of H_6 (4.08 and 3.51 to 3.81 ppm) revealed very little change in the shielding effect on H_4 and both sets of H_6.


HgCN2, HgBr2, CH3CN, R.T., 82 %
80% HOAc (aq.), R.T., 51 %
HgCN2, HgBr2, CH3CN, R.T., 34 %
1. cat. NaOMe/MeOH, MeOH, R.T.
2. H2NNH2--H2O, 100% EtOH, 70°C
"quant." over 2 steps

Scheme 2.9: Synthesis of 2-aminoethyl α-D-mannopyranosyl-(1→3)-[α-D-mannopyranosyl-(1→6)]-α-D-mannopyranoside (2)

The final glycosylation was performed again using Helferich conditions.[109, 112, 113] For this reaction, the presence of the per-*O*-acetylated mannopyranose ring at the 3-position of the reducing sugar created a steric environment that facilitated the regioselective addition of the final per-*O*-acetylated mannopyranoside to the 6-position over the 4-position of the reducing sugar **42**. Once again, the bromide **35** was used as the donor. The donor dissolved in dry CH_3CN was added to the reaction flask containing the disaccharide **42**, $HgBr_2$ and $HgCN_2$ stirring in dry CH_3CN. After stirring at room temperature for 2.5 h, a trisaccharide was isolated in 34% yield over 2 steps. Though there was very little change in the position of H_6 (3.81 to 3.88-3.97 and 3.74 ppm), a new signal ($H_{1''}$, 4.87 ppm) with a coupling constant (J = 1.8 Hz) consistent with a 1,2-di-equatorial relationship between $H_{1''}$ and $H_{2''}$ was clearly evident. With HRES-MS concluding that the desired molecular weight was achieved, the final glycosylation was deemed successful; the desired α-(1→6) trisaccharide **34** had been synthesized.

The trisaccharide **34** was then deprotected in two steps. All the esters (8 acetates and 1 benzoate) were removed by using Zemplén conditions followed by removal of the phthalimido ring with hydrazine-hydrate in anhydrous ethanol at reflux temperature. The Ligand for Con A (**2**) was isolated in a quantitative yield over 2 steps. All three of the H_1 signals were conserved (5.11, 4.91 and 4.73 with J = 1.5 Hz) along with verification of the desired molecular weight from HRES-MS.

2.4 Synthesis of the Ligand for CT (3)

The Ligand for CT (3) was the final target synthesized. As displayed in the retrosynthetic analysis (Figure 2.4), this was a simple process that occurred in few steps. It is important to note that the glycosylation step performed required the presence of a protecting group that would offer neighbouring group participation. The use of an acetate at the 2-position of lactose provided the anchimeric assistance needed in order to achieve the correct stereoselectivity; the use of acetates also offered effortless deprotection in the final few steps.

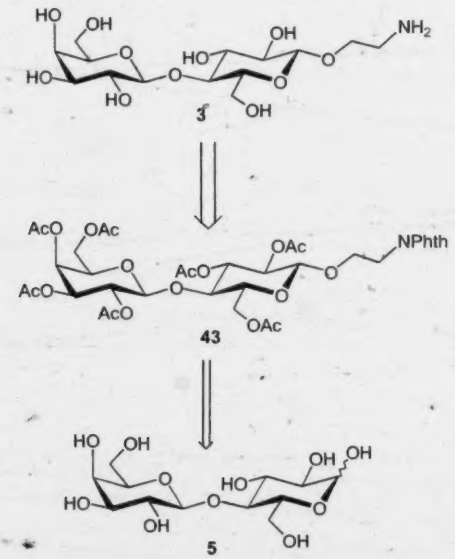


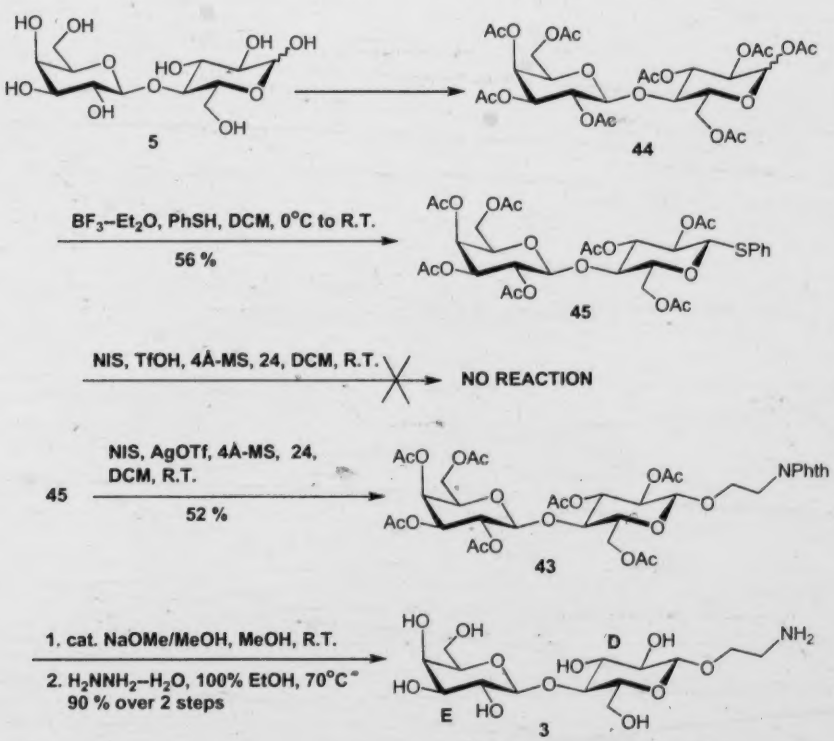
Figure 2.4: Retrosynthetic analysis of ligand for CT (3)

2.4.1 Synthesis of 2-aminoethyl β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (**3**)

The acetylation of lactose (β-D-galactopyranosyl-(1→4)-D-glucopyranose) had been performed by a previous member of the Hindsgaul lab. The availability of copious amounts of the correct β-anomer **44** simply facilitated the completion of the proposed synthesis. Conversion of the β-anomeric acetate **44** into a phenylthio glycoside **45** was achieved by the slow addition of BF_3-Et_2O into a solution of **44** and thiophenol in DCM. After stirring for 2.5 h followed by purification, the desired β-thioglycoside **45** was isolated in 56% yield. Analysis of the ^{1}H-NMR spectrum of the new product revealed that there had been a upfield shift in H_1 (5.64 to 4.64 ppm). Conservation of the 1,2-diaxial relationship between H_1 and H_2 (7.8 Hz to 10.2 Hz) confirmed that the phenyl thioglycoside had formed with the desired β-stereoselectivity. The final glycosylation was performed by the addition of catalytic trifluoromethanesulphonic acid into slurry containing **45**, **24**, 4Å-molecular sieves and *N*-iodosuccinimide in DCM at room temperature. However, the glycosylation did not occur. Repeated attempts using sub-stoichiometric amounts of triflic acid did not produce enough of the desired β-glycosylated product **43** (Scheme 2.10). It has been reported that the use of silver trifluoromethanesulphonate is simply a masked triflic acid.[109, 112] The use of 0.6 equivalents AgOTf in this glycosylation reaction did produce **43** in 52% yield. The ^{1}H-NMR spectrum displayed the slight upfield shift of H_1 (4.64 to

4.44 ppm) while maintaining its 1,2-di-axial relationship (J = 7.8 Hz) with H_2. Therefore the disaccharide **43** had been formed with β-stereoselectivity.

Deprotection of the disaccharide was achieved in two steps. The first was the use of Zemplén de-acetylation to remove all 7 acetates followed by *N*-phthalimido cleavage. Hydrazine-hydrate and the hepta-ol in anhydrous ethanol were stirred at reflux temperatures for 4 h. The desired ligand was isolated in 90% yield over 2 steps. Both H_1 signals were conserved (Ring D – H_1, 4.55 and Ring E – H_1, 4.45 ppm; J = 8.0 Hz for both); HRES-MS provided additional proof that the unprotected disaccharide **3** had been synthesized.



Scheme 2.10: Synthesis of 2-aminoethyl β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (3)

2.5 Syntheses of Linkers

The design of the synthetic route for both of the linkers would be the same. Wong and co-workers had published the synthesis of both of these linkers in their work on the use of Sialyl Lewis X to examine the inhibition of both E- and P-Selectins.[124] The synthetic design incorporating the linkers was simplistic relying on a small number of steps in order to achieve the desired linkers. Wong and co-workers stopped at the synthesis of the di-acids **46** and **47**. Each of the di-acids was coupled to a primary amine attached to the same ligand (Sialyl Lewis X). This resulted in the formation of a divalent ligand which presented Sialyl Lewis X at both ends of the linker. However, the present project was designed to place different ligands at the termini of the linker. Therefore, as displayed in the retro-synthetic analysis (Figure 2.5), benzylation was used to protect one end of the linker. The synthesized ligands would be sequentially attached to the linker using amide coupling reactions.

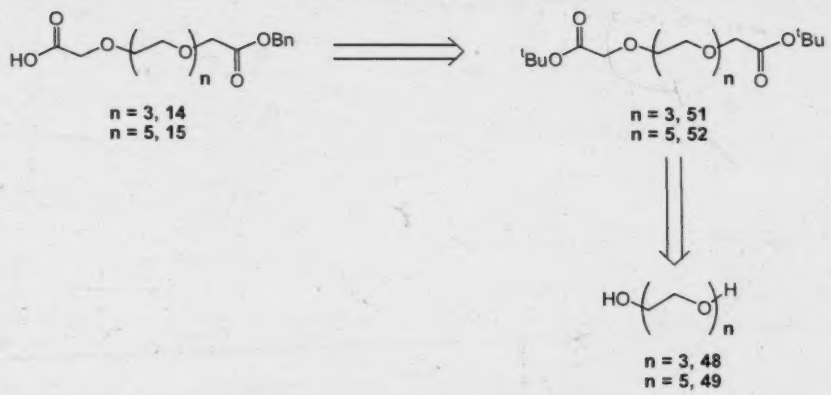


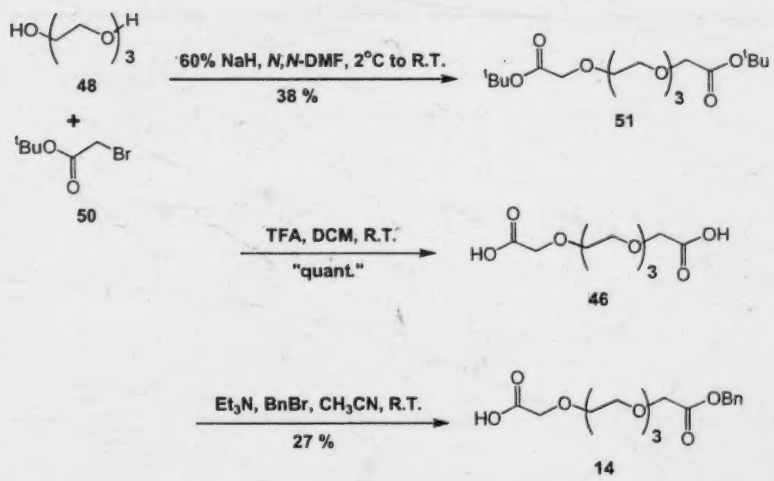
Figure 2.5: Retrosynthetic analysis of linkers

2.5.1 Synthesis of 3,6,9,12-tetraoxatetradecane-1,14-dioic acid-14-benzyl ester (**14**)

The synthesis of the linker **14** began from triethylene glycol (**48**, Scheme 2.11). Sodium hydride (60% dispersion in mineral oil) was added slowly to a solution of **48** stirred in DMF at 2°C; the reaction was allowed to warm to room temperature for 2 h. Once the heterogeneous mixture had become uniform, the reaction flask was chilled from room temperature to 2°C. A solution of tert-butylbromoacetate (**50**) in DMF was added slowly to the reaction flask.[124] After purification, the desired di-tert-butyl ester **51** was isolated in 38% yield. The critical piece of evidence from the ^{1}H-NMR spectrum was the integral ratio of the -O-C$\underline{H}_2$CO$_2$^{t}Bu signal (3.97 ppm, 4 H) relative to the -O-C$\underline{H}_2$-C$\underline{H}_2$-O- signal (3.62 to 3.67 ppm, 12 H). This confirmed that both ends of the triethylene glycol had indeed reacted with tert-butylbromoacetate; HRES-MS solidified the analysis.

The di-ester **51** was easily de-protected in the presence of trifluoroacetic acid in DCM at room temperature.[124] The di-acid **46** was obtained in a quantitative yield. Here the integral ratio between -O-C$\underline{H}_2$CO$_2$H (4.15 ppm, 4 H) and -O-C$\underline{H}_2$-C$\underline{H}_2$-O- (3.64 to 3.74 ppm, 12 H) was conserved. Mono-benzylation was attempted on several occasions by adding benzyl bromide to a solution of **46** and DBU in dry CH_3CN at room temperature. The use of DBU as the base was not beneficial; the DBU behaved as a nucleophile, displacing the bromide rather than abstracting the proton from the carboxylic acid.

In a paper published by Kim and co-workers[125], triethylamine was used as a base in order to abstract the carboxylic acid proton producing the ester. The replacement of DBU with triethylamine as the base afforded the desired product **14** in 27% yield. The ^{1}H-NMR spectrum confirmed the mono-benzylation; relative to the benzyl aromatic peak (7.34 ppm) the integral ratio for O-C$\underline{H}_2$CO$_2$Bn (4.20 ppm) and O-C$\underline{H}_2$CO$_2$H (4.18 ppm) was 5:2. HRES-MS provided further evidence that mono-benzylation had occurred; therefore, the desired linker **14** had been synthesized.

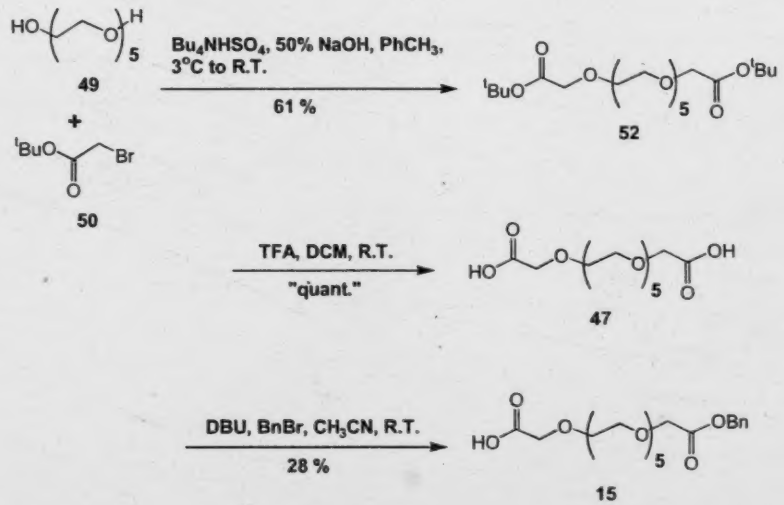


Scheme 2.11: Synthesis of 3,6,9,12-tetraoxatetradecane-1-ic acid-14-benzyl ester (14)

2.5.2 Synthesis of 3,6,9,12,15,18-hexaoxaeicosane-1-ic acid-20-benzyl ester (**15**)

To construct the other linker **15**, the synthetic scheme used was very similar to the synthesis of **14** (Scheme 2.12). The first step was to stir from 3°C to room temperature, a mixture of pentaethylene glycol (**49**), 50% NaOH, tetrabutylammonium hydrogen sulphate and **50** in toluene.[124] This produced the desired di-tert-butyl ester **52** in 61% yield. Comparison of the integrals of -O-C$\underline{H}_2$CO$_2$^{t}Bu (4.00 ppm, 4 H) and O-C$\underline{H}_2$-C$\underline{H}_2$-O (3.63 to 3.70 ppm, 20 H) gave proof that both ends of the glycol had reacted with tert-butyl bromoacetate. Once again, stirring in a solution of trifluoroacetic acid in DCM at room temperature[124]

for 1.5 h gave the di-acid **47** in a quantitative yield. The integrals for the di-acid were conserved – O-C<u>H</u>$_2$CO$_2$H (4.20 ppm, 4 H) and O-C<u>H</u>$_2$-C<u>H</u>$_2$-O (3.76 to 3.77 ppm, 4 H and 3.68 to 3.71 ppm, 16 H). Mono-benzylation of the di-acid proceeded much more smoothly using DBU and benzyl bromide in CH_3CN at room temperature. The mono-benzylated linker **15** was isolated in 28% yield. HRES-MS analysis exhibited the expected molecular weight for a mono-benzylated product. The ^{1}H-NMR spectrum confirmed this finding. The integral ratio of the benzyl aromatic peak (7.36 ppm, 5 H) to the O-C<u>H</u>$_2$-C<u>H</u>$_2$-O peak (3.68 to 3.63 ppm, 20 H) was consistent along with the presence of two separate O-C<u>H</u>$_2$-CO$_2$H and O-C<u>H</u>$_2$-CO$_2$Bn (4.20 and 4.09 ppm, 4 H) peaks.



Scheme 2.12: Synthesis of 3,6,9,12,15,18-hexaoxaeicosane-1-ic acid-20-benzyl ester (15)

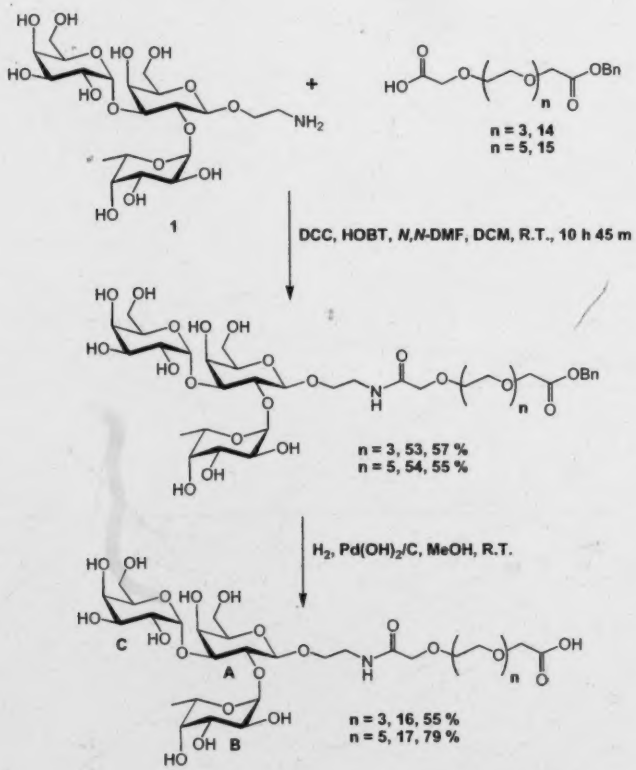
2.6 Coupling of The Ligands

2.6.1 Amide Bonding of **1** to Both Sets of Linkers (**14** and **15**)

The ligands were covalently bonded to each end of both sets of linkers in the following fashion. The first step was to covalently bond the HBG-B. That was first attempted by making the *N*-hydroxysuccinimide ester.[124] Addition of oxalyl chloride to the carboxylic acids (**14** and **15** in separate reaction flasks), removal of excess oxalyl chloride followed by addition of *N*-hydroxysuccinimide should have resulted in the formation of the reactive *N*-hydroxysuccinimide esters, respectively. However after many repeated trials, the reactive esters did not form. Simply reacting either of the acid chlorides with **1** also did not produce the amide products.

A new effort to synthesize the amide bond was made based upon the work made by Stoddart and co-workers.[126] Simple wedges containing α-mannopyranosides with free primary amines were bonded to carboxylic acids bound to a linker using DCC/HOBT. The couplings would easily produce large dendrimers with terminal α-mannopryanoside units. Using the general procedure provided, **1** was reacted with **14** and **15** individually in the presence of DCC and HOBT. Both of the reactions were stirred at room temperature for nearly 11 h, producing **53** and **54** in 57% and 55% yields respectively. Comparison of the benzyl aromatic peak to each of the H_1s revealed that there was a 5:1 ratio in both trisaccharides (**53** and **54**); the amide coupling was successful.

Hydrogenation using $Pd(OH)_2/C$ in MeOH at room temperature removed the benzyl protecting group. The free acids **16** and **17** were isolated in 55% and 79% yields respectively (Scheme 2.12); HRES-MS confirmed the molecular weights of **16** and **17**.



Scheme 2.13: Bonding of linkers to HBG-B (1)

Comparison of the ^1H- and ^{13}C-NMR spectra of all four molecules synthesized (**53**, **54**, **16** and **17**) to the free ligand **1**, revealed that the positions of all H_1s and C_1s had been conserved – Table 2.1. Of the three H_1s, one has a β–configuration while the other two have α-configurations. The proton signal at

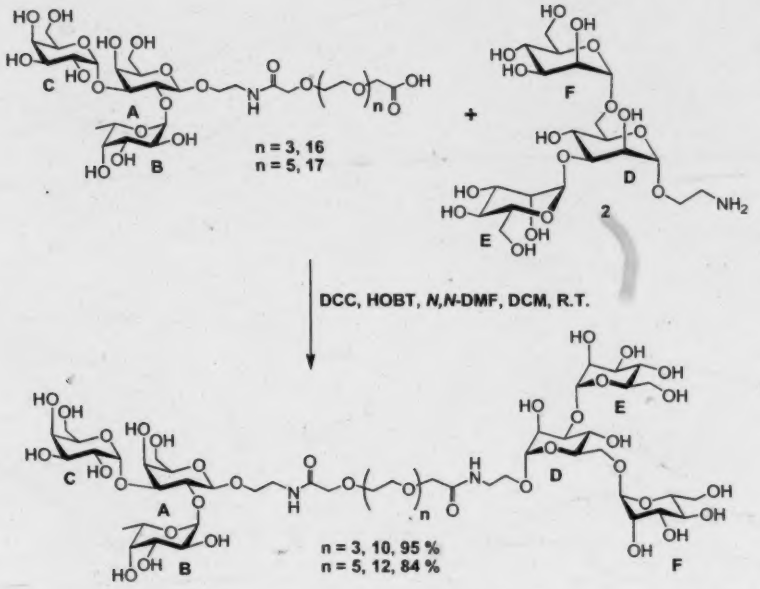
4.63 ppm for **1** displayed a coupling constant of 8.0 Hz. Examining the structure of **1**, there is only one ring (Ring A) that has its H_1 in an axial orientation; therefore a β-anomer. Using HMQC spectroscopy, the corresponding C_1 signal (102.4 ppm) was easily ascertained. The location of the signal was consistent with the empirical rule that anomeric carbon signals with β-configuration generally reside at or upfield of 100 ppm.[109] Once **1** was bound to the linkers (**14** and **15**), an upfield shift (from 4.63 ppm) was observed for both sets of Ring A-H_1s. De-benzylation produced no change in the chemical shift for the Ring A-H_1s. There was no chemical shift change observed for the carbon signal when coupling to the linker or by de-benzylation. Of the remaining two α-H_1s, the ^{1}H-NMR spectra did not reveal their specific identities; the HMBC spectrum did not clarify the situation. However, examination of the ^{1}H-NMR spectra of the linked and de-benzylated products (**16** and **17**) revealed that the remaining two α-H_1s had two different chemical shifts (5.24 and 5.26 ppm) along with different coupling constants (J = 3.5 and 2.5 Hz). The fucopyranose ring generally exhibits a greater coupling constant.[127] Therefore the peak at 5.24 ppm was assigned to Ring B-H_1; Ring C-H_1 was attributed to the signal at 5.26 ppm. Using HMQC spectroscopy, the corresponding carbon signals were ascertained (Table 2.1). While Ring B-C_1 was not affected by the coupling to the linker or by de-benzylation, Ring C-C_1 had shifted upfield when coupled to the linker.

Table 2.1: Summary of Proton and Carbon Signals (ppm) of the HBG-B – Free (1) vs. Bonded to Linker

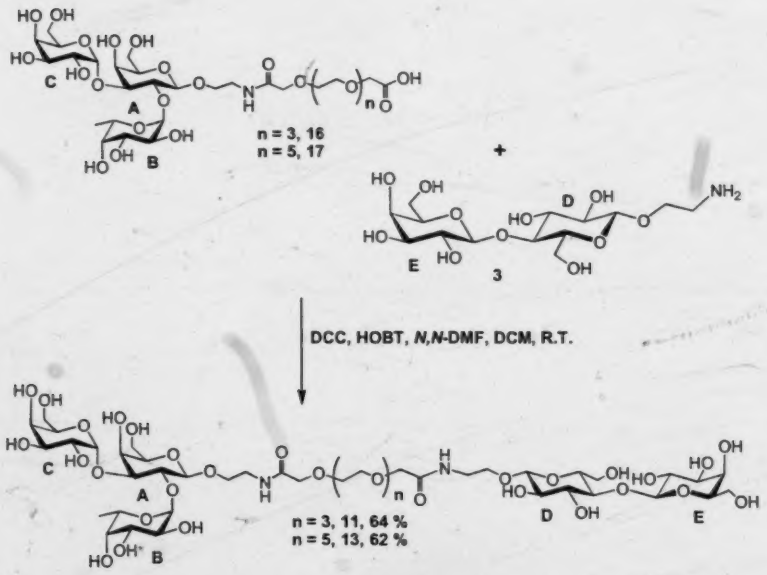
	Ring A - H_1	Ring A – C_1	Ring B - H_1	Ring B – C_1	Ring C - H_1	Ring C - C_1
1	**4.63**	**102.4**	**5.24**	**94.0**	**5.24**	**100.0**
53	**4.56**	**102.3**	**5.25**	**93.9**	**5.25**	**99.5**
54	**4.57**	**102.3**	**5.24**	**93.9**	**5.26**	**99.5**
16	**4.58**	**102.3**	**5.24**	**93.9**	**5.26**	**99.5**
17	**4.58**	**102.3**	**5.24**	**93.9**	**5.26**	**99.5**

2.6.2 Synthesis of Divalent Oligosaccharides

The final four molecules were also synthesized using DCC and HOBT.[126] Both **16** and **17** were reacted with **2** and **3**. Two portions of **16** were reacted with **2** and **3** in separate reaction flasks in the presence of DCC and HOBT at room temperature (Scheme 2.14). A pair of large molecules that contained di-valent ligands was synthesized; the HBG-B linked to the Ligand for Con A (**10**, 95%) and the HBG-B linked to the Ligand for CT (**11**, 64%). This method was repeated using **17**. Two portions of **17** were also reacted with **2** and **3** respectively producing a similar pair of large molecules (**12**, 84%) and (**13**, 62%) simply with a longer linker (Scheme 2.15).



Scheme 2.14: Linking of HBG-B (1) to the ligand for Con A (2)



Scheme 2.15: Linking of HBG-B (1) to the ligand for CT (3)

A comparison of the H_1 and C_1 signals of each of the four divalent ligands (**10**, **11**, **12** and **13**) to their free counterparts (**1**, **2** and **3**) confirmed that the amide couplings had minimal effect on their chemical environments (Tables 2.2 to 2.4). As stated earlier, there were upfield shifts for both the Ring A-H_1 and Ring C-C_1 signals when **1** was coupled to both linkers **14** and **15**. Coupling the ligands **2** and **3** to **16** and **17** respectively did not produce any change of the NMR signals (Table 2.2). The reactive centers were distant such that the chemical environments of H_1 and C_1 of the **1** fragment of **16** and **17** were unaffected.

Table 2.2: Summary of Proton and Carbon Signals (ppm) of the HBG-B – Free (1) vs. Bound in Divalent Form

	Ring A - H_1	Ring A - C_1	Ring B – H_1	Ring B - C_1	Ring C - H_1	Ring C - C_1
1	**4.63**	**102.4**	**5.24**	**94.0**	**5.24**	**100.0**
16	**4.58**	**102.3**	**5.24**	**93.9**	**5.26**	**99.5**
10	**4.58**	**102.4**	**5.24**	**94.0**	**5.26**	**99.6**
11	**4.57**	**102.4**	**5.23**	**94.0**	**5.26**	**99.6**
17	**4.58**	**102.3**	**5.24**	**93.9**	**5.26**	**99.5**
12	**4.58**	**102.4**	**5.24**	**94.0**	**5.26**	**99.6**
13	**4.58**	**102.4**	**5.24**	**94.0**	**5.26**	**99.6**

Analysis of the ^{1}H-NMR spectrum for **2** displayed the presence of three α-H_1 signals with similar coupling constants (J = 1.5 Hz); the HMQC spectrum revealed the $^{1}J_{CH}$ correlations. However, no specific assignments could be made. Upon closer inspection of **2**, the following observations were made:

- Ring D-H_1 has $^{3}J_{CH}$ to O-$\underline{C}H_2CH_2$-N and Ring D-C_3 and C_5
- Ring E-H_1 has $^{3}J_{CH}$ to Ring D-C_3 and Ring E-C_3 and C_5
- Ring F-H_1 has $^{3}J_{CH}$ to Ring D-C_6 and Ring F-C_3 and C_5

Only one of the three H_1s has a correlation to a methine carbon – Ring E-H_1. HMBC spectrum revealed that the signal at 5.09 ppm belonged to the Ring E-H_1; its corresponding C_1 signal was 103.3 ppm (Table 2.3). Of the five methylene carbons in **2**, their chemical shifts were assigned in the following manner. The peak at 39.9 ppm was assigned to O-CH_2-$\underline{C}H_2$-N; the location of this signal has been conserved throughout the synthetic schemes. Of the 3 C_6 signals, Ring D-C_6 would have a different chemical shift – further downfield – because of glycosylation at its oxygen. Assessment of the ^{13}C-NMR spectrum revealed that of the remaining four methylene peaks, two (located at 61.9 and 61.8 ppm) were closely adjacent to each other; most probably the Rings E and F-C_6 signals. Therefore, the two remaining peaks located at 66.0 and 64.3 ppm must belong to Ring D-C_6 and O-$\underline{C}H_2$-CH_2-N. Since both of the corresponding oxygens are glycosylated, removal of all protecting groups would not affect these linkages. Hence, the chemical shifts of Ring D-C_6 and O-$\underline{C}H_2$-CH_2-N should not change. Comparison of the ^{13}C-NMR spectra of **34** and **2** revealed that the

chemical shifts of Ring D-C_6 (66.2 to 66.0 ppm) and O-$\underline{C}H_2$-CH_2-N (62.1 to 64.3 ppm) were conserved. Referring back to the HMBC spectrum, the Ring D-C_6 was found to correlate to the peak located at 4.91 ppm; O-$\underline{C}H_2$-CH_2-N correlated to the peak located at 4.87 ppm. Therefore the peak at 4.91 ppm belonged to Ring F-H_1 while the peak at 4.87 ppm belonged to Ring D-H_1; HMQC spectrum displayed the chemical shifts of the corresponding Rings D and F-C_1 signals (Table 2.3).

Another important factor to consider was the effect of glycosylations at the 3 and 6-positions of Ring D upon the chemical shifts of Ring D-H_3 and H_6(s) signals. They should be downfield relative to Rings E and F-H_3 and H_6(s).[109] The use of both GCOSY and GTOCSY did not facilitate the clear identification of Ring D-H_6(s). However, as expected Ring D-H_3 was furthest upfield of the three H_3 signals.

The locations of each of the three H_1 and C_1 signals were conserved when **2** was coupled to **16** and **17** separately (Table 2.3).

Table 2.3: Summary of Proton and Carbon Signals (ppm) of the Ligand for Con A– Free (2) vs. Bound in Divalent Form

	Ring D - H_1	Ring D - C_1	Ring E – H_1	Ring E - C_1	Ring F - H_1	Ring F - C_1
2	4.87	100.9	5.11	103.3	4.91	100.3
10	4.84	100.7	5.09	103.3	4.89	100.3
12	4.83	100.7	5.09	103.3	4.89	100.3

Examination of the ^{1}H-NMR spectrum for **3** revealed that there were two β-H_1 signals (J = 8.0 Hz). The two C_1 signals were downfield of 100 ppm (102.8 and 103.8 ppm). Comparisons of the ^{1}H and ^{13}C-NMR spectra revealed that attaching **3** to **16** and **17** had an upfield shift (of 0.07 ppm) for one of its H_1s and a downfield shift (of 0.4 ppm) for one of its C_1 signals. Closer inspection of **3** displayed that though there were two β-H_1 signals, they belonged to two different monosaccharide portions – galactopyranose and glucopyranose. When performing the TCOSY, the "mix time" parameter was adjusted to discriminate the $H_1 \rightarrow H_6$ correlations of galactopyranose from glucopyranose; in effect, the $H_1 \rightarrow H_6$ correlations of galactopyranose would not be measured. Using this modified technique, the peak at 4.55 ppm was assigned to Ring D-H_1; Ring E-H_1 was located at 4.45 ppm. Using HMQC spectroscopy, the assignments of the

Rings D and E-C_1 signals were realized (Table 2.4). The HMBC spectrum confirmed the $^3J_{CH}$ between Ring D-H_1 and O-$\underline{C}H_2$-CH_2-N and Ring E-H_1 and Ring D-C_4.

Table 2.4: Summary of Proton and Carbon Signals (ppm) of the Ligand for CT – Free (3) vs. Bound in Divalent Form

	Ring D - H_1	Ring D - C_1	Ring E - H_1	Ring E - C_1
3	4.55	102.8	4.45	103.8
11	4.48	103.2	4.43	103.8
13	4.49	103.2	4.44	103.8

In all cases, the integral ratios between all protons were 1:1. LRES-MS analyses of all four sets of di-valent ligands generated measured values similar to the calculated.

2.7 Experimental

General Methods and Materials

All solvents were dried under reflux for a minimum period of 1 h prior to use in reactions. The drying agents used were CaH_2 (pyridine, CH_3CN, DCM,

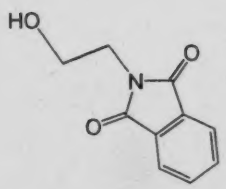
1,2-Dichloroethane, Toluene), Mg (MeOH) and Na/benzophenone (THF and Et_2O). Molecular sieves (4Å) were crushed then heated for a minimum of 4 hours at 160°C prior to use in reactions. Thin-layer chromatography was performed on Silica Gel 60-F_{254}; detection using high frequency ultra-violet light and/or charring with 5% H_2SO_4 in EtOH were the methods of choice. Column chromatography was performed using Silica Gel 60 (E. Merck, 40-63 μm). Gel filtrations were carried out using Sephadex-G10 and G15 beads.

Nuclear Magnetic Resonance analyses was performed using Varian spectrometers with frequencies ranging from 500 (Inova and Unity) to 600 (Inova) MHz in deuterated solvents ($CDCl_3$, C_6D_6 and D_2O). The temperature for all experiments was 27.0°C. All ^{13}C-NMR experiments (at 125 MHz) were performed exclusively on a Varian Inova 500 MHz spectrometer. All correlation and ^{1}H-NMR experiments were performed on all three spectrometers. The error in integration is ± 10%. In all cases, the integral values were rounded to the nearest whole number. All ^{1}H chemical shifts were reported to an error of ± 0.01 ppm; ^{13}C signals were reported to an error of ± 0.1 ppm. The coupling constants were reported to an error of ± 0.1 Hz.

Mass spectrometric analysis was performed by positive mode electrospray ionization on either a Micromass ZabSpec Hybrid Sector-TOF or a PerSeptive Biosystems Mariner Biospectrometry Workstation. A suitable liquid carrier solution was infused into the electrospray source by means of a syringe pump at a flow rate of 15 μL / m. A small amount of the sample, enough to produce a concentration in the low nanogram / μL range, was dissolved in the same

solution and introduced via a 1 or 2 μL-loop-injector. Prepurified nitrogen was used as a spray pneumatic aid and bath gas.

N-phthalimidoethanol (**24**)



A solution of ethanolamine (22g, 0.36 mol) in toluene (500 mL) was stirred at r.t. To this solution, solid phthalic anhydride (75 g, 0.51 mol) was added. The powder quickly dissolved into the solution. The round bottom flask was heated to reflux. A Dean-Stark apparatus was used to remove water from the reaction. After 15 h, the solution was concentrated and co-evaporated with $CHCl_3$. TLC and LR-ES revealed that some acetylation of the alcohol had occurred. The crude solid was dissolved in DCM (100 mL) and CH_3OH (150 mL). $NaOCH_3$ in CH_3OH (17 mL, 0.5 M) was added to the solution; the pH was 8. After two h, the reaction was neutralized (to pH 5) with Amberlite IR-120 (H^+). Concentration followed by crystallization from $CHCl_3$ afforded 34.4 g (50%, R_f = 0.43 in 1:1 EtOAc : $PhCH_3$) of a white solid.

^{1}H-NMR ($CDCl_3$ – 400 MHz) – δ 7.81 to 7.84 (m, 2 H, phthalimido –C<u>H</u>-CH-CH-C<u>H</u>-), 7.68 to 7.71 (m, 2 H, phthalimido –CH-C<u>H</u>-C<u>H</u>-CH-), 3.84 to 3.89 (m, 4 H, -O-C<u>H</u>$_2$-C<u>H</u>$_2$-N-), 1.6 (broad singlet, 1 H, OH)

^{13}C-NMR ($CDCl_3$ – 400 MHz) – δ 168.8 (s', 2 phthalimido <u>C</u>=O), 134.1 (d', phthalimido -CH-<u>C</u>H-<u>C</u>H-CH-), 131.9 (s', 2 phthalimido ring carbons), 123.4 (d', phthalimido –<u>C</u>H-CH-CH-<u>C</u>H-), 61.0 (t', O-<u>C</u>H$_2$-CH$_2$-N), 40.8 (t', -O-CH$_2$-<u>C</u>H$_2$-N-)

HR-ESMS for $C_{10}H_9NO_3Na$ ($M + Na^+$) – Calculated 214.0480; Found 214.0481

1,2,3,4,6-Penta-O-acetyl-β-D-galactopyranose (**25**)

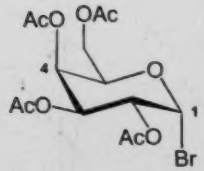
D–galactopyranose (**22**, 50 g, 0.28 mol) was stirred in acetic anhydride (150 mL, 1.59 mol) at r.t. Pyridine (20 mL, 0.25 mol) was added to the reaction. After 24 h, 50 mL acetic anhydride was added. After 72 h, 25 mL pyridine was added. 50 mL pyridine was added at 100 h. After 24 hrs, the reaction was concentrated then co-evaporated with $PhCH_3$. The residue was dissolved in diethyl ether and washed with 2N HCl, H_2O, $NaHCO_3$ (sat., aq.) and brine. After drying over Na_2SO_4, the solution was concentrated to afford the crude product. Recrystallization using hexane : Et_2O followed by drying in a desiccator over 7 days afforded 70.7 g (65%, R_f = 0.36 in 3:1:1 hexane : EtOAc : acetone) of the desired product.

^{1}H-NMR ($CDCl_3$ – 500 MHz) – δ 5.68 (d, 1H, J = 8.5 Hz, H_1), 5.40 (d, 1 H, J = 3.5 Hz, H_4), 5.32 (dd, 1 H, J = 10.5 Hz, 8.5 Hz, H_2), 5.05 (dd, 1 H, J = 10.5 Hz, 3.5 Hz, H_3), 4.14 (dd, 1 H, J = 11.3 Hz, 6.5 Hz, H_6), 4.09 (dd, 1 H, J = 11.3 Hz, 6.5 Hz, H_6), 4.03 (t, 1 H, J = 6.5 Hz, H_5), 2.15, 2.10, 2.02 & 1.98 (each s, 3 H, $COCH_3$)

^{13}C-NMR ($CDCl_3$ – 125 MHz) – δ 170.3, 170.1, 169.9 & 168.9 (each s', $COCH_3$), 92.2 (d', C_1), 71.7 (d', C_5), 70.8 (d', C_3), 67.8 (d', C_2), 66.8 (d', C_4), 61.0 (t', C_6), 20.8, 20.6, 20.6 & 20.5 (each q', $COCH_3$)

HR-ESMS for $C_{16}H_{22}O_{11}Na$ ($M + Na^+$) – Calculated 413.1060; Found 413.1066

2,3,4,6-Tetra-O-acetyl-α-D-galactopyranosyl bromide (**26**)



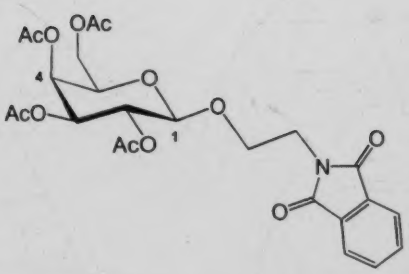
To a chilled solution of the D-galactopyranose **25** (1.02 g, 2.61 mmol) dissolved in dry DCM (5 mL), was added a solution of HBr/HOAc (33% HBr in HOAc, 3 mL) under chilled conditions. The reaction was allowed to warm to r.t. After 2 h, chloroform was added to the reaction, concentrated then co-evaporated with toluene until most of the HBr/HOAc had disappeared. The oil was then diluted with chloroform and washed with $NaHCO_3$ (sat., aq.) and brine. After drying over Na_2SO_4, the solution was concentrated and the oil (1.03 g, 96%, R_f = 0.47 in 3:1:1 hexane : EtOAc : acetone) obtained was used immediately.

^{1}H-NMR ($CDCl_3$ – 500 MHz) – δ 6.70 (d, 1 H, J = 4.0 Hz, H_1), 5.52 (d, 1 H, J = 3.0 Hz, H_4), 5.41 (dd, 1 H, J = 10.5 Hz, 3.0 Hz, H_3), 5.05 (dd, 1 H, J = 10.5 Hz, 4.0 Hz, H_2), 4.49 (t, 1 H, J = 6.8 Hz, H_5), 4.18 (dd, 1 H, J = 11.5 Hz, 6.8 Hz, H_6), 4.10 (dd, 1 H, J = 11.5 Hz, 6.8 Hz, H_6), 2.15, 2.12, 2.06 & 2.01 (each s, 3 H, $COCH_3$)

^{13}C-NMR ($CDCl_3$ – 125 MHz) – δ 170.2, 170.0, 169.8 & 169.7 (each s', $COCH_3$), 88.1 (d', C_1), 71.0 (d', C_5), 68.0 (d', C_3), 67.8 (d', C_2), 67.0 (d', C_4), 60.8 (t', C_6), 20.7, 20.6, 20.5 & 20.5 (each q', $COCH_3$)

HR-ESMS for $C_{14}H_{19}O_9Na^{79}Br$ ($M + Na^+$) – Calculated 433.0105; Found 433.0106

2-Phthalimidoethyl 2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside (**27**)



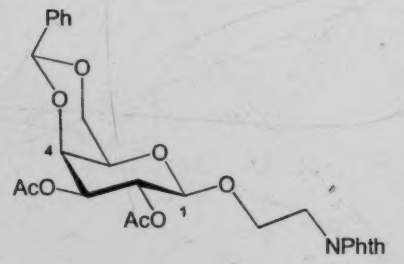
Under inert atmosphere **24** (387 mg, 2.03 mmol), $HgCN_2$ (403 mg, 1.59 mmol) and $HgBr_2$ (623 mg, 1.73 mmol) were stirred in dry DCM (6 mL). To the slurry was added slowly, **26** (713 mg obtained from 513 mg acetyl 2,3,4,6-tetra-*O*-acetyl galactopyranoside, **25**) dissolved in 5 mL dry DCM, at r.t. The syringe was rinsed with 2 x 2 mL dry DCM. The reaction was stirred at r.t. After 18 h, the slurry was filtered over Celite and washed with chloroform. The organic solution was washed with 10% KI (aq.), H_2O and brine. After drying over Na_2SO_4, the solution was concentrated. Purification was performed by column chromatography using 8:1:1, 7:2:1 to 6:2:2 hexane : EtOAc : acetone. The desired product was isolated as a yellow oil (469 mg, 61%, R_f = 0.26 in 3:1:1 hexane : EtOAc : acetone).

^{1}H-NMR ($CDCl_3$ – 600 MHz) – δ 7.80 (dd, 2 H, J = 5.4 Hz, 3.0 Hz, phthalimido -C<u>H</u>-CH-CH-C<u>H</u>-), 7.67 (dd, 2 H, J = 5.4 Hz, 3.0 Hz, phthalimido -CH-C<u>H</u>-C<u>H</u>-CH-), 5.27 (dd, 1 H, J = 3.6 Hz, 0.6 Hz, H_4), 5.08 (dd, 1 H, J = 10.8 Hz, 7.8 Hz, H_2), 4.90 (dd, 1 H, J = 10.8 Hz, 3.6 Hz, H_3), 4.43 (d, 1 H, J = 7.8 Hz, H_1), 4.00 (m, 1 H, H_6), 3.95 (dd, 2 H, J = 6.6 Hz, 4.0 Hz, -O-C<u>H</u>$_2$-CH$_2$-N-), 3.85 (m, 2 H, -O-CH$_2$-C<u>H</u>$_2$-N-), 3.80 (m, 2 H, H_5, H_6), 2.05, 1.98, 1.89 & 1.80 (each s, 3 H, COC<u>H</u>$_3$)

^{13}C-NMR ($CDCl_3$ – 125 MHz) – δ 170.2, 170.1, 170.1 & 169.1 (each s', $\underline{C}OCH_3$), 167.9, (each s', phthalimido $\underline{C}$=O), 133.9 (d', phthalimido -CH-$\underline{C}$H-$\underline{C}$H-CH-), 132.0 (each s', 2 phthalimido ring carbons), 123.2 (d', phthalimido -$\underline{C}$H-CH-CH-$\underline{C}$H-), 100.7 (d', C_1), 70.8 (d', C_3), 70.6 (d', C_5), 68.4 (d', C_2), 66.9 (d', C_4), 65.6 (t', C_6), 61.1 (t', -O-$\underline{C}H_2$-CH_2-N-), 37.3 (t', -O-CH_2-$\underline{C}H_2$-N-), 20.5, 20.5, 20.4 & 20.3 (each q', $CO\underline{C}H_3$)

HR-ESMS for $C_{24}H_{27}NO_{12}Na$ ($M + Na^+$) – Calculated 544.1404; Found 544.1435

2-Phthalimidoethyl 2,3-di-O-acetyl-4,6-O-benzylidene-β-D-galactopyranoside (**28**)



A solution of the **27** (2.99 g, 6.24 mmol) in dry methanol (5 mL) was stirred at r.t. A solution of 0.5 M $NaOCH_3$ in dry methanol (1.2 mL, 0.6 mmol) was added to the solution at r.t.; the pH was 9. The reaction was stirred at r.t. for 7 h. Amberlite IR-120 (H^+) resin was added to the solution. Once the pH was 5, the solution was filtered over Celite then concentrated. The oil was co-evaporated with anhydrous ethanol to remove traces of methanol and water. The product obtained (3.27 g, R_f = 0.11 in 1:9 CH_3OH : $CHCl_3$) was used immediately.

The crude solid 2-phthalimidoethyl β-D-galactopyranoside (3.27 g, 9.26 mmol) was dissolved in dry DMF (5 mL) at r.t. Dimethoxytoluene (4.2 mL, 28.0 mmol) followed by camphor sulphonic acid (50 mg) were added to the solution at

r.t.; the pH was 3. The solution was stirred at r.t. After 24 h, triethylamine was added to neutralize the 10-camphorsulphonic acid and the solution was concentrated then co-evaporated with toluene several times to remove DMF and excess dimethoxytoluene.

The oil (R_f = 0.44 in 1:9 CH_3OH : $CHCl_3$) was then dissolved in pyridine (3 mL, 37.1 mmol) and acetic anhydride (2.6 mL, 27.6 mmol) at r.t. After 30 h, the reaction was concentrated then co-evaporated with toluene several times. Purification by column chromatography (eluent polarity ranging from 5% to 50% ethyl acetate in toluene) furnished a white solid (1.92 g, 59% over 3 steps, R_f = 0.38 in 1:4 EtOAc : $PhCH_3$).

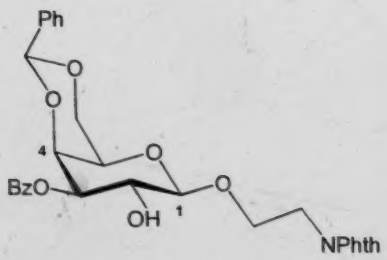
^{1}H-NMR ($CDCl_3$ – 600 MHz) – δ 7.80 (m, 2 H, phthalimido -C<u>H</u>-CH-CH-C<u>H</u>-), 7.65 (m, 2 H, phthalimido -CH-C<u>H</u>-C<u>H</u>-CH-), 7.45 (m, 2 H, benzylidene ortho C<u>H</u>), 7.33 (m, 3 H, benzylidene meta & para C<u>H</u>), 5.42 (s, 1 H, benzylidene C<u>H</u>), 5.31 (dd, 1 H, J = 10.2 Hz, 7.8 Hz, H_2), 4.87 (dd, 1 H, J = 10.2 Hz, 3.6 Hz, H_3), 4.50 (d, 1 H, J = 7.8 Hz, H_1), 3.94 (d, 1 H, J = 3.6 Hz, H_4), 4.10 (dd, 1 H, J = 12.3 Hz, 1.5 Hz, H_6), 4.05 (m, 1 H, -O-C<u>H</u>$_2$-CH$_2$-N-), 3.95 (dd, 1 H, J = 12.3 Hz, 1.5 Hz, H_6), 3.84 to 3.90 (m, 3 H, -O-CH$_2$-C<u>H</u>$_2$-N-), 3.44 (broad s, 1 H, H_5), 2.00 & 1.85 (each s, 3 H, COC<u>H</u>$_3$)

^{13}C-NMR ($CDCl_3$ – 125 MHz) – δ 170.7 & 169.1 (each s', <u>C</u>OCH$_3$), 168.0 (s', 2 phthalimido <u>C</u>=O), 137.4 (s', benzylidene ring), 133.8 (d', phthalimido –CH-<u>C</u>H-<u>C</u>H-CH-), 132.1 (s', 2 phthalimido ring carbons), 129.0 (d', benzylidene para <u>C</u>H), 128.1 (d', benzylidene meta <u>C</u>H), 126.4 (d', benzylidene ortho <u>C</u>H), 123.2 (d', phthalimido –<u>C</u>H-CH-CH-<u>C</u>H-), 101.0 (d', benzylidene <u>C</u>H), 100.4 (d', C_1), 73.2

(d', C_4), 72.0 (d', C_3), 68.7 (t', C_6), 68.1 (d', C_2), 66.4 (d', C_5), 65.1 (t', -O-$\underline{C}H_2$-CH_2-N-), 37.5 (t', -O-CH_2-$\underline{C}H_2$-N-), 20.8 & 20.5 (each q', CO$\underline{C}H_3$)

HR-ESMS for $C_{27}H_{27}NO_{10}Na$ ($M + Na^+$) – Calculated 548.1533; Found 548.1534

2-Phthalimidoethyl 3-O-benzoyl-4,6-O-benzylidene-β-D-galactopyranoside (**19**)



A solution of **28** (3.89 g, 7.4 mmol) in dry methanol (20 mL) and dry DCM (30 mL) was stirred at r.t. A solution of 0.5 M $NaOCH_3$ in dry methanol (1.5 mL, 0.76 mmol) was added to the solution at r.t.; the pH was 9. After 6 h, Amberlite IR-120 (H^+) resin was added to the reaction. Once pH was < 5, the solution was filtered over Celite and concentrated. The oil (R_f = 0.44 in 1:9 CH_3OH : $CHCl_3$) was then co-evaporated with anhydrous ethanol and chloroform to remove traces of methanol and water.

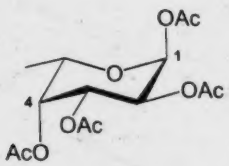
The oil was then stirred in dry dichloromethane (20 mL) at temperatures ranging from -30° to -35°C. To this solution, was added benzoyl chloride (1.5 mL, 12.9 mmol) followed by pyridine (2.1 mL, 26.0 mmol). The reaction was stirred at temperatures ranging from -30° to -35°C for 6 h. The reaction mixture was diluted with chloroform then washed with H_2O and brine then dried over Na_2SO_4. Purification by column chromatography with 5% to 50% ethyl acetate in toluene resulted in the isolation of the desired product (3.4 g, 84% over 2 steps, R_f = 0.46 in 1:1 EtOAc : $PhCH_3$).

^{1}H-NMR (C_6D_6 – 600 MHz) – δ 8.19 (d, 2 H, J = 7.8 Hz, benzoyl ortho C<u>H</u>), 7.50 (d, 2 H, J = 7.8 Hz, benzylidene ortho C<u>H</u>), 7.38 (m, 2 H, phthalimido -C<u>H</u>-CH-CH-C<u>H</u>-), 7.07 (m, 2 H, benzylidene meta C<u>H</u>), 7.04 (m, 2 H, benzoyl & benzylidene para C<u>H</u>), 6.95 (t, 2 H, J = 7.5 Hz, benzoyl meta C<u>H</u>), 6.81 (m, 2 H, phthalimido -CH-C<u>H</u>-C<u>H</u>-CH-), 5.25 (dd, 1 H, J = 13.5 Hz, 4.2 Hz, H_3), 5.13 (s, 1 H, benzylidene C<u>H</u>), 4.34 (dd, 1 H, J = 13.5 Hz, 7.8 Hz, H_2), 4.11 (d, 1 H, J = 7.8 Hz, H_1), 4.10 (d, 1 H, J = 4.2 Hz, H_4), 4.00 (m, 1 H, -O-C<u>H_2</u>-CH_2-N-), 3.90 (d, 1 H, J = 12.3 Hz, H_6), 3.63 (m, 1 H, -O-C<u>H_2</u>-CH_2-N-), 3.54 (m, 2 H, -O-CH_2-C<u>H_2</u>-N-), 3.29 (d, 1 H, J = 12.3 Hz, H_6), 2.59 (s, 1 H, H_5)

^{13}C-NMR (C_6D_6 – 125 MHz) – δ 168.4 (s', phthalimido <u>C</u>=O), 166.5 (s', benzoyl <u>C</u>=O), 138.8 (s', benzylidene ring), 133.5 (d', phthalimido –CH-<u>C</u>H-<u>C</u>H-CH-), 133.0 (d', benzoyl para <u>C</u>H), 132.4 (s', 2 phthalimido ring carbons), 130.6 (s', benzoyl ring), 130.2 (d', benzoyl ortho <u>C</u>H), 128.8 (d', benzylidene para <u>C</u>H), 128.5 (d', benzoyl meta <u>C</u>H), 128.2 (d', benzylidene ortho <u>C</u>H), 126.7 (d', benzylidene ortho <u>C</u>H), 123.1 (d', phthalimido –<u>C</u>H-CH-CH-<u>C</u>H-), 104.5 (d', C_1), 101.0 (d', benzylidene <u>C</u>H), 74.4 (d', C_3), 74.0 (d', C_4), 68.9 (d', C_2), 68.8 (t', C_6), 67.7 (t', -O-<u>C</u>H_2-CH_2-N-), 65.5 (d', C_5), 38.3 (t', -O-CH_2-<u>C</u>H_2-N-)

HR-ESMS for $C_{30}H_{27}NO_9Na$ ($M + Na^+$) – Calculated 568.1584; Found 568.1585

1,2,3,4-Tetra-O-acetyl-α-L-fucopyranose (**31**)



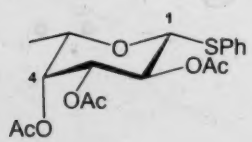
L-fucopyranose (3.6 g, 22.0 mmol) was chilled over an ice-H_2O bath. Acetic anhydride (12.4 mL, 131.5 mmol) followed by pyridine (10.6 mL, 131.2 mmol) was added to the white solid. After 17 h, the solution was concentrated then co-evaporated with toluene. Filtration over silica using toluene resulted in isolation of a white solid (7.9 g, quant., R_f = 0.46 in 1:4 EtOAc : $PhCH_3$).

^{1}H-NMR (C_6D_6 – 600 MHz) – δ 6.71 (d, 1 H, J = 3.6 Hz, H_1), 5.57 (dd, 1 H, J = 10.8 Hz, 3.6 Hz, H_2), 5.58 (dd, 1 H, J = 10.8 Hz, 3.6 Hz, H_3), 5.41 (dd, 1 H, J = 3.6 Hz, 1.5 Hz, H_4), 3.86 (dd, 1 H, J = 6.6 Hz, 1.5 Hz, H_5), 1.74, 1.67, 1.65 & 1.63 (each s, 3 H, $COCH_3$), 0.9 (d, 3 H, J = 6.6 Hz, H_6)

^{13}C-NMR (C_6D_6 – 125 MHz) – δ 170.3, 169.9, 169.7 & 168.8 (each s', $COCH_3$), 90.3 (d', C_1), 70.9 (d', C_4), 68.3 (d', C_3), 67.5 (d', C_5), 67.1 (d', C_2), 20.3, 20.3, 20.1 & 19.9 (each q', $COCH_3$), 15.8 (q', C_6)

HR-ESMS for $C_{14}H_{20}O_9NaS$ ($M + Na^+$) – Calculated 355.1005; Found 355.1008

Phenyl 2,3,4-tri-O-acetyl-1-thio-β-L-fucopyranoside (**32**)



A solution of **31** (7.9 g, 23.8 mmol) in dry DCM (20 mL) was stirred at r.t. Thiophenol (3.7 mL, 36.1 mmol) followed by BF_3—Et_2O (9.1 mL, 71.8 mmol) were added slowly to the solution placed over ice-H_2O bath. The reaction was allowed to warm to r.t.

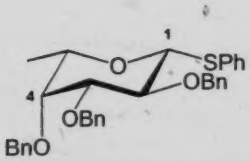
After 16 h, the reaction was quenched with triethylamine. The solution was then washed with 2N HCl, H_2O, $NaHCO_3$ (sat., aq.) and brine; dried over Na_2SO_4. Purification by column chromatography – eluent polarity ranging from 2% to 50% ethyl acetate in toluene – produced the desired beta product (7.8 g, 86%, R_f = 0.50 in 1:4 EtOAc : $PhCH_3$).

^{1}H-NMR (C_6D_6 – 600 MHz) – δ 7.55 (m, 2 H, phenyl ortho C<u>H</u>), 7.03 (m, 2 H, phenyl meta C<u>H</u>), 7.00 (m, 1 H, phenyl para C<u>H</u>), 5.54 (t, 1 H, J = 10.2 Hz, H_2), 5.18 (dd, 1 H, J = 3.6 Hz, 0.6 Hz, H_4), 5.10 (dd, 1 H, J = 10.2 Hz, 3.6 Hz, H_3), 4.52 (d, 1 H, J = 10.2 Hz, H_1), 3.00 (dd, 1 H, J = 6.0 Hz, 0.6 Hz, H_5), 1.79, 1.72 & 1.57 (each s, 3 H, COC<u>H</u>$_3$), 0.90 (d, 3 H, J = 6.0 Hz, H_6)

^{13}C-NMR (C_6D_6 – 125 MHz) – δ 170.2, 169.7 & 169.0 (each s', <u>C</u>OCH$_3$), 133.5 (s', phenyl ring), 132.8 (d', phenyl ortho <u>C</u>H), 129.0 (d', phenyl meta <u>C</u>H), 127.9 (d', phenyl para <u>C</u>H), 86.3 (d', C_1), 73.0 (d', C_3), 72.8 (d', C_5), 70.6 (d', C_4), 67.8 (d', C_2), 20.5, 20.3 & 19.9 (each q', CO<u>C</u>H$_3$), 16.3 (q', C_6)

HR-ESMS for $C_{18}H_{22}O_7NaS$ ($M + Na^+$) – Calculated 405.0984; Found 405.0984

Phenyl 2,3,4-tri-<u>O</u>-benzyl-1-thio-β-L-fucopyranoside (**20**)



A solution of **32** (7.8 g, 20.4 mmol) in dry methanol (15 mL) was stirred at r.t. A solution of 0.5 M $NaOCH_3$ in dry methanol (1.2 mL, 0.6 mmol) was added to the solution at r.t.; the pH was 9. After stirring for 18 h at r.t., Amberlite IR-120 (H^+) resin was added to the solution. Once pH was < 5, the solution was filtered over Celite

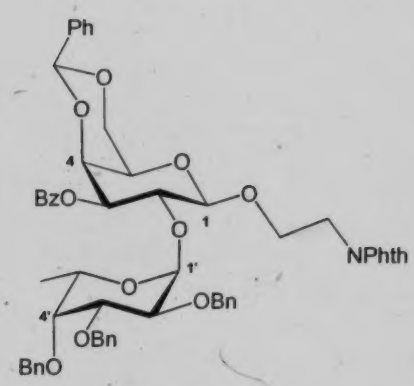
then concentrated. The oil was co-evaporated with anhydrous ethanol to remove traces of methanol and water. The solid (5.5 g, quant.) residue was used without further purification.

The solid (1.46 g, 5.7 mmol) was then dissolved in DMF at r.t. Portions of sodium hydride (1.03 g, 25.8 mmol) were added slowly to the solution at r.t. Once the exotherm had calmed, benzyl bromide (4.1 mL, 34.5 mmol) was added slowly (in order to control the exotherm) to the reaction flask. After stirring for 17 h, excess sodium hydride and methanol were added to quench excess benzyl bromide. Once pH was > 9, the solution was poured into a H_2O : EtOAc mixture. The organic layer was washed with H_2O and brine; dried over Na_2SO_4. Purification of the crude product using column chromatography – eluent polarity ranging from 0 to 5% ethyl acetate in toluene – produced a white solid (2.15 g, 72% over 2 steps, R_f = 0.80 in 1:4 EtOAc : $PhCH_3$).

^{1}H-NMR (C_6D_6 – 600 MHz) -- δ 7.74 (m, 2 H, phenyl ortho C<u>H</u>), 7.43 (m, 2 H, benzyl ortho C<u>H</u> – H_2), 7.36 (m, 2 H, benzyl ortho C<u>H</u> – H_4), 7.30 (m, 2 H, benzyl ortho C<u>H</u> – H_3), 7.16 to 7.19 (m, 6H, benzyl meta C<u>H</u>), 7.08 to 7.13 (m, 3 H, benzyl para C<u>H</u>), 7.00 to 7.02 (m, 2 H, phenyl meta C<u>H</u>), 6.93 to 6.97 (m, 1 H, phenyl para C<u>H</u>), 4.96 (d, 1 H J = 11.4 Hz, benzyl C<u>H_2</u> – H_4), 4.85 (d, 1 H, J = 10.8 Hz, benzyl C<u>H_2</u> – H_2), 4.67 (d, 1 H, J = 10.8 Hz, benzyl C<u>H_2</u> – H_2), 4.58 (d, 1 H, J = 9.6 Hz, H_1), 4.57 (d, 1 H, J = 11.7 Hz, benzyl C<u>H_2</u> – H_3), 4.51 (d, 1 H, J = 11.7 Hz, benzyl C<u>H_2</u> – H_3), 4.49 (d, 1 H, J = 11.4 Hz, benzyl C<u>H_2</u> – H_4), 4.11 (t, 1 H, 9.6 Hz, H_2), 3.31 (dd, 1 H, J = 9.6 Hz, 3.0 Hz, H_3), 3.20 (d, 1 H, J = 3.0 Hz, H_4), 2.97 (d, 1 H, J = 6.0 Hz, H_5), 1.15 (d, 3 H, J = 6.0 Hz, H_6)

^{13}C-NMR (C_6D_6 – 125 MHz) -- δ 139.5 (s', benzyl ring carbon – C_2), 139.4 (s', benzyl ring carbon – C_4), 139.1 (s', benzyl ring carbon – C_3), 135.2 (s', phenyl ring carbon), 132.1 (d', phenyl ortho CH), 129.0 (d', phenyl meta CH), 128.6, 128.5 & 128.4 (each d', benzyl meta CH), 128.2 (d', benzyl para CH), 128.0 (d', benzyl ortho CH – C_2), 127.8 (d', benzyl ortho CH – C_4), 127.7 (d', benzyl ortho CH – C_3), 127.6 & 127.6 (each d', benzyl para CH), 127.1 (d', phenyl para CH), 87.9 (d', C_1), 84.8 (d', C_3), 77.6 (d', C_2), 77.4 (d', C_4), 75.5 (t', benzyl $\underline{C}H_2$ – C_2), 75.0 (t', benzyl $\underline{C}H_2$ – C_4), 74.6 (d', C5), 72.8 (t', benzyl $\underline{C}H_2$ – C_3), 17.4 (q', C_6)
HR-ESMS for $C_{33}H_{34}O_4NaS$ ($M + Na^+$) – Calculated 549.2070; Found 549.2075

2-Phthalimidoethyl 2,3,4-tri-O-benzyl-α-L-fucopyranosyl-(1→2)-3-O-benzoyl-4,6-O-benzylidene-β-D-galactopyranoside (**30**)



A mixture of **19** (3.0 g, 5.50 mmol) and **20** (3.49 g, 6.64 mmol) were weighed then dried under vacuum together. The mixture was then stirred in dry dichloromethane (30 mL) at r.t. under inert atmospheric conditions. *N*-iodosuccinimide (2.47 g, 11.0 mmol) followed a catalytic portion of trifluoromethanesulphonic acid (3 μL) were added to the solution. After the reaction was stirred at r.t. for 6 h, triethylamine was added to the reaction. The reaction solution was then diluted with EtOAc then washed with 5% $Na_2S_2O_3$ (aq.) and brine; dried over Na_2SO_4. Purification of the crude oil

by column chromatography – eluent polarity ranging from 2% to 50% ethyl acetate in toluene – afforded a white solid (3.77 g, 71%, R_f = 0.71 in 1:1 EtOAc : $PhCH_3$).

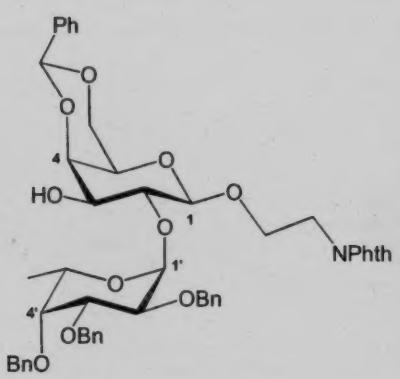
^{1}H-NMR (C_6D_6 – 600 MHz) – δ 7.58 (m, 2 H, benzylidene ortho CH), 7.46 (dd, 2 H, J = 5.4 Hz, 3.0 Hz, phthalimido –C<u>H</u>-CH-CH-C<u>H</u>-), 7.36 (m, 6 H, benzyl ortho CH), 7.07 to 7.21 (m, 17 H, benzyl meta & para, benzoyl ortho, meta & para, benzylidene meta & para C<u>H</u>), 6.87 (dd, 1 H, J = 5.4 Hz, 3.0 Hz, phthalimido – CH-C<u>H</u>-C<u>H</u>-CH-), 5.39 (d, 1 H, J = 3.6 Hz, $H_{1'}$), 5.22 (s, 1 H, benzylidene C<u>H</u>), 4.98 (d, 1 H, J = 11.4 Hz, benzyl CH_2 – $H_{4'}$), 4.74 (d, 1 H, J = 12.0 Hz, benzyl C<u>H</u>$_2$ – $H_{3'}$), 4.61 (d, 1 H, J = 10.8 Hz, benzyl C<u>H</u>$_2$ – $H_{2'}$), 4.54 (d, 1 H, J = 12.0 Hz, benzyl C<u>H</u>$_2$ – $H_{3'}$), 4.53 (d, 1 H, J = 10.8 Hz, benzyl C<u>H</u>$_2$ – $H_{2'}$), 4.52 (d, 1 H, J = 11.4 Hz, benzyl CH_2 – $H_{4'}$), 4.25 (d, 1 H, J = 7.8 Hz, H_1), 4.21 (d, 1 H, J = 6.6 Hz, $H_{5'}$), 4.18 (dd, 1 H, J = 10.2 Hz, 3.6 Hz, $H_{2'}$), 4.09 (dt, 1 H, J_d = 10.2 Hz, J_t = 6.0 Hz, -O-C<u>H</u>$_2$-CH_2-N-), 4.05 (dd, 1 H, J = 7.8 Hz, 2.4 Hz, H_2), 4.03 (dd, 1 H, J = 10.2 Hz, 3.0 Hz, $H_{3'}$), 3.90 (dt, 1 H, J_d = 10.2 Hz, J_t = 6.0 Hz, -O-C<u>H</u>$_2$-CH_2-N-), 3.77 to 3.85 (m, 3 H, H_6 & -O-CH_2-C<u>H</u>$_2$-N-), 3.59 (m, 2 H, H_3 & H_4), 3.40 (d, 1 H, J = 3.0 Hz, $H_{4'}$), 3.26 (dd, 1 H, J =12.0 Hz, 1.8 Hz, H_6), 2.46 (s, 1 H, H_5), 1.22 (d, 1 H, J = 6.6 Hz, $H_{6'}$)

^{13}C-NMR (C_6D_6 – 125 MHz) – δ 168.0 (s', benzoyl and 2 phthalimido <u>C</u>=O), 139.7 (s', 2 benzyl ring carbons – $C_{3'}$ & $C_{4'}$), 138.9 (s', benzoyl ring carbon), 138.8 (s', benzyl ring carbon – $C_{2'}$), 133.4 (d', phthalimido –CH-<u>C</u>H-<u>C</u>H-CH-), 132.7 (s', 2 phthalimido ring carbons), 128.7, 128.5, 128.5, 128.4, 128.3, 128.3 & 127.6 (each d', benzyl ortho, meta & para, benzoyl ortho, meta & para,

benzylidene meta & para CH), 127.0 (d', benzylidene ortho CH), 123.0 (d', phthalimido –CH-CH-CH-CH-), 101.9 (d', C_1), 101.5 (d', benzylidene CH), 99.1 (d', $C_{1'}$), 79.7 (d', $C_{3'}$), 79.0 (d', $C_{4'}$), 78.1 (d', $C_{2'}$), 77.9 (d', C_2), 75.3 (t', benzyl CH_2 – $C_{4'}$), 73.7 (d', C_3), 73.6 (t', benzyl CH_2 – $C_{2'}$), 73.2 (t', benzyl CH_2 – $C_{3'}$), 68.9 (t', C_6), 67.4 (d', $C_{5'}$), 66.7 (d', C_5), 65.5 (t', -O-CH_2-CH_2-N-), 37.9 (t', -O-CH_2-CH_2-N-), 16.9 (q', $C_{6'}$)

HR-ESMS for $C_{57}H_{55}NO_{13}Na$ ($M + Na^+$) – Calculated 984.3571; Found 984.3566

2-Phthalimidoethyl 2,3,4-tri-O-benzyl-α-L-fucopyranosyl-(1→2)-4,6-O-benzylidene-β-D-galactopyranoside (**33**)



A solution of **30** (3.77 g, 3.95 mmol) in dry methanol (20 mL) and dry DCM (3 mL) was stirred at r.t. A solution of 0.5 M $NaOCH_3$ in dry methanol (1.6 mL, 0.8 mmol) was added to the solution at r.t.; the pH was 9. After 5 h, Amberlite IR-120 (H^+) resin was added to the reaction. Once pH was < 5, the solution was filtered over Celite and concentrated. The solid was then co-evaporated with anhydrous ethanol and chloroform to remove traces of methanol and water. A white solid was obtained (2.78 g, 83%, R_f = 0.5 in 1:1 EtOAc : $PhCH_3$).

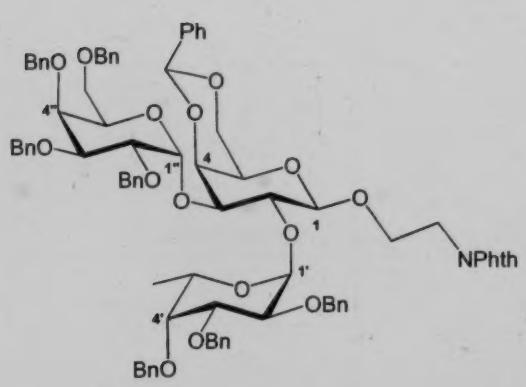
^{1}H-NMR (C_6D_6 – 600 MHz) – δ 7.60 (m, 2 H, benzylidene ortho CH), 7.46 (dd, 2 H, J = 5.4 Hz, 3.0 Hz, phthalimido –CH-CH-CH-CH-), 7.36 (m, 6 H, benzyl ortho

CH), 7.07 to 7.21 (m, 12 H, benzyl meta & para & benzylidene meta & para CH), 6.86 (dd, 2 H, J = 5.4 Hz, 3.0 Hz, phthalimido –CH-CH-CH-CH-), 5.40 (d, 1 H, J = 3.6 Hz, $H_{1'}$), 5.21 (s, 1 H, benzylidene CH), 4.98 (d, 1 H, J = 11.4 Hz, benzyl CH_2 – $H_{4'}$), 4.74 (d, 1 H, J = 12.0 Hz, benzyl CH_2 – $H_{3'}$), 4.61 (d, 1 H, J = 10.8 Hz, benzyl CH_2 – $H_{2'}$), 4.55 (d, 1 H, J = 12.0 Hz, benzyl CH_2 – $H_{3'}$), 4.55 (d, 1 H, J = 10.8 Hz, benzyl CH_2 – $H_{2'}$), 4.52 (d, 1 H, J = 11.4 Hz, benzyl CH_2 – $H_{4'}$), 4.25 (d, 1 H, J = 7.8 Hz, H_1), 4.20 (dd, 1 H, J = 6.6 Hz, 0.6 Hz, $H_{5'}$), 4.19 (dd, 1 H, J = 10.2 Hz, 3.6 Hz, $H_{2'}$), 4.09 (dt, 1 H, J_d = 10.2 Hz, J_t = 6.0 Hz, -O-CH_2-CH_2-N-), 4.05 (dd, 1 H, J = 10.2 Hz, 3.0 Hz, $H_{3'}$), 4.04 (dd, 1 H, J_d = 10.2 Hz, 7.8 Hz, H_2), 3.90 (dt, 1 H, J_d = 10.2 Hz, J_t = 6.0 Hz, -O-CH_2-CH_2-N-), 3.78 to 3.85 (m, 3 H, H_6 & -O-CH_2-CH_2-N-), 3.59 (m, 2 H, H_3 & H_4), 3.40 (dd, 1 H, J = 3.0 Hz, 0.6 Hz, $H_{4'}$), 3.26 (dd, 1 H, J = 12.0 Hz, 1.8 Hz, H_6), 2.46 (s, 1 H, H_5), 1.22 (d, 3 H, J = 6.6 Hz, $H_{6'}$)

^{13}C-NMR (C_6D_6 – 125 MHz) – δ 168.0 (s', 2 phthalimido C=O), 139.7 (s', benzyl ring carbon – $C_{4'}$), 138.8 (s', benzyl ring carbon – $C_{3'}$), 138.7 (s', benzyl ring carbon – $C_{2'}$), 133.5 (d', phthalimido –CH-CH-CH-CH-), 132.7 (s', 2 phthalimido ring carbons), 127.6 to 128.7 (each d', benzyl meta & para & benzylidene meta & para CH), 127.0 (d', benzylidene ortho CH), 123.0 (d', phthalimido –CH-CH-CH-CH-), 101.9 (d', C_1), 101.5 (d', benzylidene CH), 99.2 (d', $C_{1'}$), 79.7 (d', $C_{3'}$), 78.9 (d', $C_{4'}$), 78.0 & 78.0 (each d', C_2 & $C_{2'}$), 75.7 (d', C_4), 75.3 (t', benzyl CH_2 – $C_{4'}$), 73.7 (d', C_3), 73.6 (t', benzyl CH_2 – $C_{2'}$), 73.1 (t', benzyl CH_2 – $C_{3'}$), 68.9 (t', C_6), 67.4 (d', $C_{5'}$), 66.7 (d', C_5), 65.5 (t', -O-CH_2-CH_2-N-), 37.9 (t', -O-CH_2-CH_2-N-), 16.9 (q', $C_{6'}$)

HR-ESMS for $C_{50}H_{51}NO_{12}Na$ ($M + Na^+$) – Calculated 880.3309; Found 880.3309

2-Phthalimidoethyl 2,3,4-tri-O-benzyl-α-L-fucopyranosyl-(1→2)-[2,3,4,6-tetra-O-benzyl-α-D-galactopyranosyl-(1→3)]-4,6-O-benzylidene-β-D-galactopyranoside (**18**)



The pre-dried disaccharide **33** (1.60 g, 1.87 mmol) was stirred in dichloromethane (25 mL) at r.t. in the presence of powdered 4Å molecular sieves (ca. 0.5 g) for 1 hour. DMF (1.4 mL, 18.1 mmol) followed by $CuBr_2$ (2.09 g, 9.37 mmol) were added to the solution at r.t. After 0.5 h, **21** (1.82 g, 2.82 mmol) was added to the reaction flask at r.t. The reaction was stirred for 18 h at r.t. The reaction was filtered over Celite, washing with chloroform. The organic solution was then washed with 6 N HCl, H_2O, $NaHCO_3$ (sat.) and brine; dried over Na_2SO_4. The crude material was purified by column chromatography – eluent polarity ranging from 8 : 0.75 : 0.75 to 6 : 2 : 2 hexane : EtOAc : acetone – afforded a white solid (1.36 g, 53%, R_f = 0.36 in 3:1:1 hexane : EtOAc : acetone + 1% Et_3N).

^{1}H-NMR (C_6D_6 – 600 MHz) – δ 7.47 (m, 2 H, benzylidene ortho C<u>H</u>), 7.43 (m, 4 H, benzyl ortho C<u>H</u> & phthalimido –C<u>H</u>-CH-CH-C<u>H</u>-), 7.31 to 7.38 (m, 10 H, benzyl ortho C<u>H</u>), 7.04 to 7.21 (m, 21 H, benzyl meta & para C<u>H</u>), 6.92 to 7.00

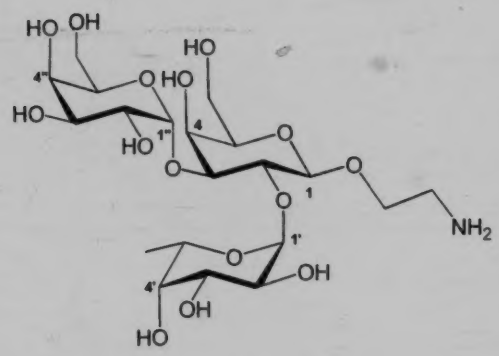
(m, 4 H, benzylidene meta & para C<u>H</u>), 6.78 (dd, 2 H, J = 5.4 Hz, 3.0 Hz, phthalimido –CH-C<u>H</u>-C<u>H</u>-CH-), 5.80 (d, 1 H, J = 3.6 Hz, $H_{1'}$), 5.45 (d, 1 H, J = 3.6 Hz, $H_{1''}$), 5.15 (s, 1 H, benzylidene C<u>H</u>), 5.07 (d, 1 H, J = 11.4 Hz, benzyl C<u>H_2</u> – $H_{4''}$), 4.91 (d, 1 H, J = 11.4 Hz, benzyl C<u>H_2</u> – $H_{4'}$), 4.75 (d, 1 H, J = 11.4 Hz, benzyl C<u>H_2</u> – $H_{3'}$), 4.72 (d, 1 H, J = 12.3 Hz, benzyl C<u>H_2</u> – $H_{2'}$), 4.69 (d, 1 H, J = 12.3 Hz, benzyl C<u>H_2</u> – $H_{3''}$), 4.62 (d, 1 H, J = 11.4 Hz, benzyl C<u>H_2</u> – $H_{3'}$), 4.59 (d, 1 H, J = 12.3 Hz, benzyl C<u>H_2</u> – $H_{3''}$), 4.58 (d, 1 H, J = 10.8 Hz, benzyl C<u>H_2</u> – $H_{2''}$), 4.56 (d, 2 H, J = 11.4 Hz, benzyl C<u>H_2</u> – $H_{4'}$ & benzyl C<u>H_2</u> – $H_{4''}$), 4.43 (m, 3 H, $H_{5''}$, H_2 & $H_{5'}$), 4.37 (dd, 1 H, J = 9.6 Hz, 3.6 Hz, $H_{2''}$), 4.36 (d, 1 H, J = 12.3 Hz, benzyl C<u>H_2</u> – $H_{2'}$), 4.33 (d, 1 H, J = 12.0 Hz, benzyl C<u>H_2</u> – $H_{6''}$), 4.32 (d, 1 H, J = 10.8 Hz, benzyl C<u>H_2</u> – $H_{2''}$), 4.29 (d, 1 H, J = 12.0 Hz, benzyl C<u>H_2</u> – $H_{6''}$), 4.24 (d, 1 H, J = 7.2 Hz, H_1), 4.21 (dd, 1 H, J = 9.6 Hz, 3.0 Hz, $H_{3''}$), 4.19 (dd, 1 H, J = 7.8 Hz, 2.4 Hz, $H_{3'}$), 4.13 (m, 1 H, -O-C<u>H_2</u>-CH_2-N-), 4.08 (dd, 1 H, J = 7.8 Hz, 3.6 Hz, $H_{2'}$), 4.07 (dd, 1 H, J = 3.9 Hz, 1.5 Hz, H_4), 4.02 (dd, 1 H, J = 9.6 Hz, 3.9 Hz, H_3), 3.91 (dd, 1 H, J = 12.3 Hz, 1.2 Hz, H_6), 3.82 to 3.87 (m, 2 H, -O-C<u>H_2</u>-CH_2-N- & $H_{6''}$), 3.81 (m, 1 H, -O-CH_2-C<u>H_2</u>-N-), 3.73 (m, 1 H, -O-CH_2-C<u>H_2</u>-N-), 3.71 (d, 1 H, J = 3.0 Hz, $H_{4''}$), 3.62 (dd, 1 H, J = 2.4 Hz, 0.6 Hz, $H_{4'}$), 3.36 (dd, 1 H, J = 9.6 Hz, 4.8 Hz, $H_{6''}$), 3.23 (dd, 1 H, J = 12.3 Hz, 1.2 Hz, H_6), 2.58 (s, 1 H, H_5), 1.31 (d, 3 H, J = 6.6 Hz, $H_{6'}$)

^{13}C-NMR (C_6D_6 – 125 MHz) – δ 168.0 (s', 2 phthalimido <u>C</u>=O), 139.9, 139.9, 139.7, 139.6, 139.6, 139.2, 139.2 & 138.5 (each s', benzyl and benzylidene ring carbons), 133.4 (d', phthalimido –<u>C</u>H-CH-CH-<u>C</u>H-), 132.7 (s', 2 phthalimido ring carbons), 128.6, 128.6, 128.5, 128.5, 128.5, 128.4, 128.4, 128.3, 127.9, 127.7,

127.6, 127.6 & 126.9 (each d', benzyl and benzylidene ortho, meta & para CH), 123.0 (d', phthalimido –CH-CH-CH-CH-), 103.0 (d', C_1), 101.5 (d', benzylidene CH), 97.8 (d', $C_{1'}$), 92.3 (d', $C_{1''}$), 80.2 (d', $C_{3'}$), 79.1 (d', $C_{4'}$), 78.1 (d', $C_{3''}$), 77.4 (d', $C_{2''}$), 77.4 (d', $C_{2'}$), 76.7 (d', $C_{4''}$), 75.4 (t', benzyl CH_2 – $C_{4'}$), 75.3 (d', C_3), 75.0 (t', benzyl CH_2 – $C_{4''}$), 73.5 (d', benzyl CH_2 – $C_{3''}$), 73.3 (d', C_2), 73.3 (t', benzyl CH_2 – $C_{6''}$), 73.3 (t', benzyl CH_2 – $C_{2'}$), 73.0 (t', benzyl CH_2 – $C_{3'}$), 71.6 (d', C_4), 71.5 (t', benzyl CH_2 – $C_{2''}$), 71.0 (t', $C_{6''}$), 70.7 (d', $C_{5''}$), 69.1 (t', C_6), 67.1 (d', $C_{5'}$), 66.5 (t', -O-CH_2-CH_2-N-), 66.4 (d', C_5), 38.1 (t', -O-CH_2-CH_2-N-), 17.1 (q', $C_{6'}$)

LR-ESMS for $C_{84}H_{85}NO_{17}Na$ ($M + Na^+$) – Calculated 1403.6; Found 1403.7

2-Aminoethanol α-L-fucopyranosyl-(1→2)-[α-D-galactopyranosyl-(1→3)]-β-D-galactopyranoside (**1**)



The protected trisaccharide **18** (808 mg, 0.59 mmol), was stirred in 100% EtOH (20 mL) at r.t. After the addition of H_2NNH_2—H_2O (35% wt in H_2O, 4 mL, 43.8 mmol), the reaction was stirred under reflux (95°C) for 3.5 h. The reaction was concentrated then co-evaporated with 100% EtOH. The residue (933 mg, R_f = 0.96 in 4:1 CH_3CN : H_2O) was used immediately.

The residue was stirred gently in 1:1 EtOAc : 100% EtOH (10 mL). $Pd(OH)_2/C$ (1.15 g) was added to the slurry. A balloon of H_2 was used to flush

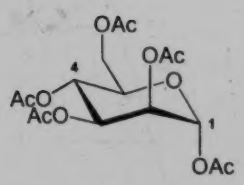
out air. Balloon pressure of H_2 was applied to the reaction for a period of 8 d. The reaction was filtered over Celite then concentrated. Purification using Sephadex G-10 gel filtration afforded a white solid (230 mg, 74% over 2 steps, R_f = 0.37 in 1:2:1 ^{n}BuOH : ^{n}PrOH : 0.1 M HCl).

^{1}H-NMR (D_2O – 500 MHz) – δ 5.24 (d, 1 H, J = 3.5 Hz, $H_{1'}$), 5.24 (d, 1 H, J = 3.5 Hz, $H_{1''}$), 4.63 (d, 1 H, J = 8.0 Hz, H_1), 4.33 to 4.25 (m, 5 H), 4.08 to 3.71 (m, 22 H), 3.31 to 3.17 (m, 4 H), 1.21 (d, 3 H, J = 6.5 Hz, $H_{6'}$)

^{13}C-NMR (D_2O – 500 MHz) – δ 102.4 (d', C_1), 100.0 (d', $C_{1''}$), 94.0 (d', $C_{1'}$), 76.6, 75.4, 75.0, 72.7, 72.0, 70.6, 70.2, 70.2, 70.1, 68.9, 68.7 & 67.8 (each d'), 67.1 (t'), 64.2 (d'), 62.1 & 61.9 (each t'), 40.7 (t', -O-CH_2-CH_2-N-), 16.1 (q', $C_{6'}$)

HR-ESMS for $C_{20}H_{37}NO_{15}Na$ ($M + Na^+$) – Calculated 554.2055; Found 554.2055

1,2,3,4,6-Penta-O-acetyl-α-D-mannopyranose (**38**)



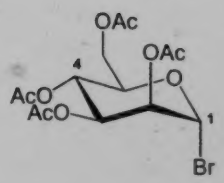
D-mannopyranose (**37**, 10.0 g, 55.6 mmol) was stirred in acetic anhydride (30 mL, 0.32 mol) at r.t. Pyridine (50 mL, 62 mol) followed by a few crystals of 4-dimethylamino pyridine were added to the solution at r.t. After stirring for 15 h at r.t., the reaction solvent was co-evaporated with $PhCH_3$. The residue was dissolved in EtOAc then washed with 2 N HCl, H_2O, $NaHCO_3$ (sat., aq.) and brine; dried over Na_2SO_4. The crude residue was purified over silica using toluene. A yellow oil was obtained (22.9 g, quant., R_f = 0.54 in 1:1 $PhCH_3$: EtOAc).

^{1}H-NMR ($CDCl_3$ – 500 MHz) – δ 6.06 (d, 1 H, J = 2.3 Hz, H_1), 5.32 (dd, 2 H, J = 3.8 Hz, 2.3 Hz, H_3 & H_4), 5.24 (t, 1 H, J = 2.3 Hz, H_2), 4.25 (dd, 1 H, J = 12.5 Hz, 5.0 Hz, H_6), 4.07 (dd, 1 H, J = 12.5, 2.5 Hz, H_6), 4.03 (m, 1 H, H_5), 2.15, 2.15, 2.07, 2.03 & 1.98 (each s, 3 H, $COCH_3$)

^{13}C-NMR ($CDCl_3$ – 125 MHz) – δ 170.5, 169.9, 169.6, 169.4 & 167.9 (each s', $COCH_3$), 90.5 (d', C_1), 70.5 (d', C_5), 68.7 (d', C_3), 68.3 (d', C_2), 65.5 (d', C_4), 62.0 (t', C_6), 20.7, 20.7, 20.6, 20.5 & 20.5 (each q', $COCH_3$)

HR-ESMS for $C_{16}H_{22}O_{11}Na$ ($M + Na^+$) – Calculated 413.1054; Found 413.1050

2,3,4,6-Tetra-O-acetyl-α-D-mannopyranosyl bromide (**35**)



To a chilled solution of **38** (3.28 g, 8.41 mmol) dissolved in dry DCM (10 mL), was added a solution of HBr/HOAc (33% HBr in HOAc, ca. 3.5 mL) under chilled conditions. The reaction was stirred at r.t. for 5 h. Toluene and chloroform were added to the reaction flask. The reaction was concentrated then co-evaporated with toluene until most of the HBr/HOAc had disappeared. The oil was then diluted with chloroform and washed with $NaHCO_3$ (sat., aq.) and brine. After drying over Na_2SO_4, the solution was concentrated and the oil (3.60 g, quant., R_f = 0.40 in 3:1:1 hexane : EtOAc : acetone) obtained was used immediately.

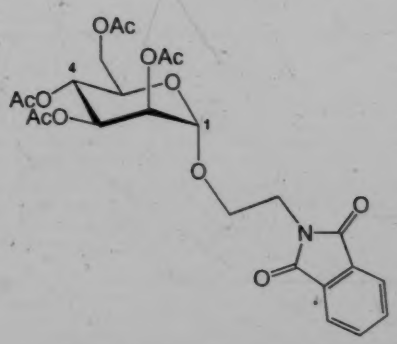
^{1}H-NMR ($CDCl_3$ – 400 MHz) – δ 6.27 (d, 1 H, J = 1.2 Hz, H_1), 5.69 (dd, 1 H, J = 10.2 Hz, 3.4 Hz, H_3), 5.42 (dd, 1 H, J = 10.2 Hz, 1.2 Hz, H_2), 5.35 (dd, 1 H, J = 10.2, 3.4 Hz, H_4), 4.31 (dd, 1 H, J = 12.4 Hz, 4.8 Hz, H_6), 4.20 (ddd, 1 H, J = 10.2

Hz, 4.8 Hz, 2.4 Hz, H_5), 4.11 (dd, 1 H, J = 12.4 Hz, 2.4 Hz, H_6), 1.99, 2.05, 2.08 & 2.15 (each s, 3 H, $COCH_3$)

^{13}C-NMR ($CDCl_3$ – 100 MHz) – δ 170.5, 169.6, 169.5 & 169.5 (each s', $COCH_3$), 83.0 (d', C_1), 72.8 (d', C_5), 72.1 (d', C_2), 67.9 (d', C_3), 65.3 (d', C_4), 61.4 (t', C_6), 20.7, 20.6, 20.6 & 20.5 (each q', $COCH_3$)

HR-ESMS for $C_{14}H_{19}O_9NaBr$ ($M + Na^+$) – Calculated 433.0105; Found 433.0100

2-Phthalimidoethyl 2,3,4,6-tetra-O-acetyl-α-D-mannopyranoside (**39**)



In an inert atmosphere was prepared a solution containing **24** (1.43 g, 7.49 mmol), $HgCN_2$ (1.52 g, 6.01 mmol) and $HgBr_2$ (2.37 g, 6.60 mmol) in dry acetonitrile (10 mL). **35** (2.04 g, 4.98 mmol) dissolved in dry acetonitrile (10 mL) was added slowly to the solution at r.t. The reaction was stirred at r.t. for 15 h. The solution was diluted with EtOAc then washed with 5% KI (aq.), H_2O and brine. After drying over Na_2SO_4, the solution was concentrated and purified by column chromatography. Eluent polarity ranging from 5% to 50% ethyl acetate in toluene was used. A white solid (1.75 g, 88%, R_f = 0.58 in 1:1 $PhCH_3$: EtOAc).

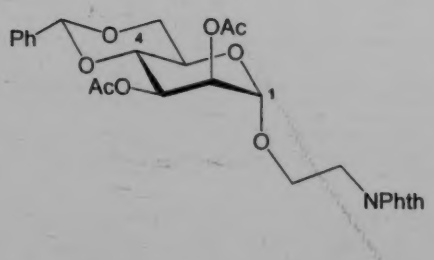
^{1}H-NMR (C_6D_6 – 600 MHz) – δ 7.46 (dd, 2 H, J = 5.4 Hz, 3.0 Hz, phthalimido -CH-CH-CH-CH-), 6.85 (dd, 2 H, J = 5.4 Hz, 3.0 Hz, phthalimido -CH-CH-CH-CH-), 5.62 (m, 2 H, H_3 and H_4), 5.21 (dd, 1 H, J = 3.0 Hz, 1.8 Hz, H_2), 4.69 (d, 1 H, J

= 1.8 Hz, H_1), 4.30 (dd, 1 H, J = 12.6 Hz, 2.7 Hz, H_6), 4.19 (dd, 1 H, J = 12.6 Hz, 1.8 Hz, H_6), 3.97 (ddd, 1 H, J = 10.7 Hz, 2.7 Hz, 1.8 Hz, H_5), 3.62 (m, 2 H, -O-$C\underline{H}_2$-$C\underline{H}_2$-N-), 3.50 (dd, 1 H, J = 8.7 Hz, 2.7 Hz, -O-CH_2-$C\underline{H}_2$-N-), 3.29 (dd, 1 H, J = 9.6 Hz, 4.8 Hz, -O-$C\underline{H}_2$-CH_2-N-), 1.80, 1.67, 1.61 & 1.61 (each s, 3 H, $COC\underline{H}_3$)

^{13}C-NMR (C_6D_6 – 125 MHz) – δ 170.0, 169.5, 169.4 & 169.3 (each s', $\underline{C}OCH_3$), 167.8 (s', 2 phthalimido $\underline{C}$=O), 133.6 (d', phthalimido -CH-$\underline{C}$H-$\underline{C}$H-CH-), 132.5 (s', 2 phthalimido ring carbons), 123.2 (d', phthalimido -$\underline{C}$H-CH-CH-$\underline{C}$H-), 97.5 (d', C_1), 69.9 (d', C_2), 69.7 (d', C_5), 69.7 (d', C_3), 66.4 (d', C_4), 64.5 (t', -O-$\underline{C}H_2$-CH_2-N-), 62.5 (t', C_6), 36.9 (t', -O-CH_2-$\underline{C}H_2$-N-), 20.4, 20.3 & 20.2 (each q', $CO\underline{C}H_3$)

HR-ESMS for $C_{24}H_{27}NO_{12}Na$ ($M + Na^+$) – Calculated 544.1426; Found 544.1436

2-Phthalimidoethyl 2,3-di-O-acetyl-4,6-O-benzylidene-α-D-mannopyranoside (**40**)



A solution of **39** (1.16 g, 2.23 mmol) in dry methanol (5 mL) was stirred at r.t. A solution of 0.5 M $NaOCH_3$ in dry methanol (0.5 mL, 0.25 mmol) was added to the solution at r.t.; the pH was 9. The solution was stirred at r.t. for 22 h. Amberlite IR-120 (H^+) resin was added to the solution. Once pH was < 5, the solution was filtered over Celite then concentrated. The oil was co-evaporated with anhydrous ethanol to remove traces of methanol and water. A residue (0.8 g, R_f = 0.20 in 8.8:1:0.2 DCM : CH_3OH : H_2O) was isolated and used without further purification.

The solid residue (0.8 g, 2.27 mmol) was dissolved in dry dichloromethane (10 mL) and dry acetonitrile (10 mL) at r.t. Dimethoxytoluene (0.175 mL, 1.17 mmol) followed by camphor sulphonic acid (10 mg) were added to the solution at r.t.; the pH was 3. After 8 h, an additional 0.175 mL of dimethoxytoluene was added. The solution was stirred at r.t. After several hours, TLC revealed that the reaction was complete. Triethylamine was added to neutralize the camphor sulphonic acid and the solution was concentrated then co-evaporated with toluene several times to remove excess dimethoxytoluene. The crude material isolated (1.75 g, 0.44 mmol) was used without further purification.

The crude material (1.75 g, 3.97 mmol) was then dissolved in pyridine (2 mL, 24.8 mmol) and acetic anhydride (2 mL, 21.2 mmol) at r.t. After 1.5 h, the reaction was simply concentrated then co-evaporated with toluene several times. Purification by column chromatography (eluent polarity ranging from 9:0.5:0.5 to 6:2:2: hexane : EtOAc : acetone) afforded the desired product as a white solid (395 mg, 34% over 3 steps, R_f = 0.31 3:1:1 hexane : EtOAc : acetone).

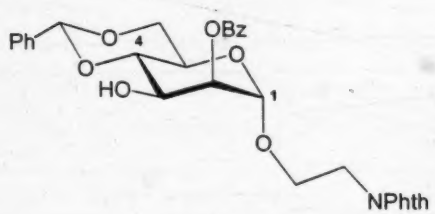
^{1}H-NMR (C_6D_6 – 600 MHz) – δ 7.46 (m, 2 H, benzylidene ortho C<u>H</u>), 7.45 (dd, 2 H, J = 5.4 Hz, 3.0 Hz, phthalimido -C<u>H</u>-CH-CH-C<u>H</u>-), 7.11 (m, 3 H, benzylidene meta & para C<u>H</u>), 6.85 (dd, 2 H, J = 5.4 Hz, 3.0 Hz, phthalimido -CH-C<u>H</u>-C<u>H</u>-CH-), 5.73 (dd, 1 H, J = 10.8 Hz, 3.6 Hz, H_3), 5.67 (dd, 1 H, J = 3.6 Hz, 1.8 Hz, H_2), 5.32 (s, 1 H, benzylidene C<u>H</u>), 4.71 (d, 1 H, J = 1.8 Hz, H_1), 4.15 (dd, 1 H, J = 15.0 Hz, 10.8 Hz, H_4), 4.09 (dd, 1 H, J = 10.2 Hz, 4.8 Hz, H_6), 3.98 (ddd, 1 H, J = 15.0 Hz, 10.2 Hz, 4.8 Hz, H_5), 3.62 (m, 2 H, -O-C<u>H</u>$_2$-C<u>H</u>$_2$-N-), 3.57 (triplet, 1 H, J

= 10.2 Hz, H_6), 3.45 (m, 1 H, -O-CH_2-$C\underline{H}_2$-N-), 3.29 (dd, 1 H, J = 10.2 Hz, 5.4 Hz, -O-$C\underline{H}_2$-CH_2-N-), 1.74 & 1.61 (each s, 3 H, $COC\underline{H}_3$)

^{13}C-NMR (C_6D_6 -- 125 MHz) – δ 169.2 & 169.1 (each s', $\underline{C}OCH_3$), 167.9 (s', 2 phthalimido $\underline{C}$=O), 138.1 (s', benzylidene ring carbon), 133.6 (d', phthalimido -CH-$\underline{C}$H-$\underline{C}$H-CH-), 132.5 (s', 2 phthalimido ring carbons), 129.1 (d', benzylidene para $\underline{C}$H), 128.3 (d', benzylidene meta $\underline{C}$H), 126.9 (d', benzylidene ortho $\underline{C}$H), 123.2 (d', phthalimido -$\underline{C}$H-CH-CH-$\underline{C}$H-), 102.5 (d', benzylidene $\underline{C}$H), 98.4 (d', C_1), 76.6 (d', C_4), 70.6 (d', C_2), 68.9 (d', C_3), 68.8 (t', C_6), 64.7 (d', C_5), 64.2 (t', -O-$\underline{C}H_2$-CH_2-N-), 36.9 (t', -O-CH_2-$\underline{C}H_2$-N-), 20.4 & 20.3 (each q', CO$\underline{C}H_3$)

HR-ESMS for $C_{27}H_{27}NO_{10}Na$ ($M + Na^+$) – Calculated 548.1533; Found 548.1536

2-Phthalimidoethyl 2-O-benzoyl-4,6-O-benzylidene-α-D-mannopyranoside (**36**)



A solution of **40** (1.24 g, 2.36 mmol) in dry methanol (5 mL) and dry DCM (5 mL) was stirred at r.t. A solution of 0.5 M $NaOCH_3$ in dry methanol (0.47 mL, 0.24 mmol) was added to the solution at r.t.; the pH was 9. The solution was stirred at r.t. for 5 h. Amberlite IR-120 (H^+) resin was added to the solution. Once pH was < 5, the solution was filtered over Celite then concentrated. The solid was co-evaporated with anhydrous ethanol to remove traces of methanol. The solid (1.13 g, R_f = 0.18 in 1:1 EtOAc: $PhCH_3$) obtained was used without further purification.

A solution of 2-phthalimidoethyl 4,6-*O*-benzylidene-α-D-mannoside (1.13 g, 2.56 mmol) in DMF (5 mL) was prepared at r.t. Trimethylorthobenzoate (1.2 mL, 7.00 mmol) followed by camphor sulphonic acid (ca. 10 mg) were added to the solution at r.t.; the pH was 3. The reaction was stirred for 2 d. Triethylamine was added to neutralize camphor sulphonic acid and the solution was concentrated then co-evaporated with toluene to remove excess trimethylorthobenzoate. Purification by column chromatography – eluent polarity ranging from 5% to 50% ethyl acetate in toluene – produced the desired 2-*O*-benzoyl product (676 mg, 52% over 2 steps, R_f = 0.70 in 1:1 EtOAc : $PhCH_3$).

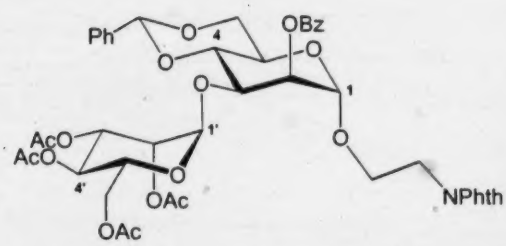
^{1}H-NMR (C_6D_6 – 500 MHz) – δ 8.19 (d, 2 H, J = 7.5 Hz, benzoyl ortho CH), 7.48 (dd, 2 H, J = 5.5 Hz, 3.0 Hz, phthalimido -C<u>H</u>-CH-CH-C<u>H</u>-), 7.42 (dd, 2 H, J = 8.0 Hz, 1.5 Hz, benzylidene ortho CH), 7.15 (m, 3 H, benzylidene meta & para CH), 7.10 (m, 1 H, benzoyl para CH), 7.06 (m, 2 H, benzoyl meta CH), 6.89 (m, 2 H, phthalimido -CH-C<u>H</u>-C<u>H</u>-CH-), 5.62 (m, 1 H, H_2), 5.29 (d, 1 H, J = 2.5 Hz, benzylidene C<u>H</u>), 4.82 (d, 1 H, J = 1.8 Hz, H_1), 4.29 (broad peak, 1 H, H_3), 4.07 (dd, 1 H, J = 10.2 Hz, 4.8 Hz, H_6), 4.02 (m, 1 H, H_4), 3.79 (ddd, 1 H, J = 9.8 Hz, 5.0 Hz, 1.0 Hz, H_5), 3.72 (2nd order m, 2 H, -O-C<u>H</u>$_2$-C<u>H</u>$_2$-N-), 3.53 (dt, 1 H, J_t = 10.2 Hz, J_d = 1.0 Hz, H_6), 3.49 (m, 1 H, -O-C<u>H</u>$_2$-CH$_2$-N-), 3.30 (m, 1 H, -O-CH$_2$-C<u>H</u>$_2$-N-), 2.70 (broad s, 1 H, C_3-O<u>H</u>)

^{13}C-NMR (C_6D_6 – 125 MHz) – δ 168.0 (s', 2 phthalimido <u>C</u>=O), 165.9 (s', benzoyl <u>C</u>=O), 138.3 (s', benzylidene ring carbon), 133.7 (d', phthalimido –CH-<u>C</u>H-<u>C</u>H-CH-), 133.1 (d', benzoyl para <u>C</u>H), 132.4 (s', benzoyl ring carbons), 130.6 (s', 2 phthalimido ring carbons), 130.2 (d', benzoyl ortho <u>C</u>H), 129.0, 128.6 & 128.3

(each d', benzylidene meta & para and benzoyl meta CH), 126.9 (d', benzylidene ortho CH), 123.2 (d', phthalimido –CH-CH-CH-CH-), 102.1 (d', benzylidene CH), 98.6 (d', C_1), 79.6 (d', C_2), 68.8 (t', C_6), 67.7 (d', C_3), 64.4 (t', -O-CH_2-CH_2-N-), 64.3 (d', C_5), 37.4 (t', -O-CH_2-CH_2-N-)

HR-ESMS for $C_{30}H_{27}NO_9Na$ ($M + Na^+$) – Calculated 568.1583; Found 568.1582

2-Phthalimidoethyl 2-O-benzoyl-4,6-O-benzylidene-2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl-(1→3)-α-D-mannopyranoside (**41**)



To a chilled solution of **38** (1.54 g, 3.95 mmol) dissolved in dry dichloromethane (10 mL), was added a solution of HBr/HOAc (33% HBr in HOAc, ca. 4 mL) under chilled conditions. The reaction was allowed to warm to r.t. and stirred for 2 h. Toluene and chloroform were added to the reaction flask. The reaction was concentrated then co-evaporated with toluene until most of the HBr/HOAc had disappeared. The oil was then diluted with chloroform and washed with $NaHCO_3$ (sat., aq.) and brine. After drying over Na_2SO_4, the solution was concentrated; the oil (2.60 g, R_f = 0.39 in 3:1:1 hexane : EtOAc : acetone) obtained was used immediately.

In an inert atmosphere was prepared a slurry containing **36** (1.54 g, 2.83 mmol), $HgCN_2$ (858 mg, 3.39 mmol) and $HgBr_2$ (1.23 g, 3.43 mmol) in dry acetonitrile (6 mL). **35** (2.60 g, 6.34 mmol) dissolved in dry acetonitrile (1 mL)

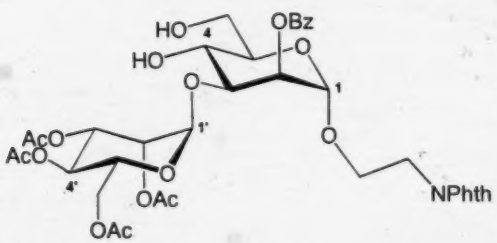
was added slowly to the slurry at r.t. The syringe was rinsed with dry acetonitrile (3 x 1 mL). The reaction was stirred at r.t. for 2.5 h. The reaction was concentrated then co-evaporated with $CHCl_3$. The residue was dissolved in $CHCl_3$: 10% KI (aq.); the organic phase was washed with 10% KI (aq.), $NaHCO_3$ (sat., aq.) H_2O and brine. After drying over Na_2SO_4, the solution was concentrated and purified by column chromatography. Eluent polarity ranging from 8:1:1 to 4:3:3 hexane : EtOAc : acetone was used. The desired disaccharide was obtained as a white solid (2.02 g, 82%, R_f = 0.31 in 3:1:1 hexane : EtOAc : $PhCH_3$).

^{1}H-NMR (C_6D_6 – 600 MHz) – δ 8.40 (m, 2 H, benzoyl ortho C<u>H</u>), 7.54 (d, 4 H, J = 7.8 Hz, benzylidene ortho C<u>H</u> & phthalimido –C<u>H</u>-CH-CH-C<u>H</u>-), 7.26 (t, 2 H, J = 7.8 Hz, benzylidene meta C<u>H</u>), 7.20 (t, 2 H, J = 7.8 Hz, benzoyl meta C<u>H</u>), 7.16 (m, 2 H, benzoyl & benzylidene para C<u>H</u>), 6.89 (dd, 2 H, J = 5.4 Hz, 3.0 Hz, phthalimido -CH-C<u>H</u>-C<u>H</u>-CH-), 5.80 (t, 1 H, J = 9.9 Hz, $H_{4'}$), 5.77 (dd, 1 H, J = 3.9 Hz, 1.5 Hz, H_2), 5.75 (dd, 1 H, J = 9.9 Hz, 3.0 Hz, $H_{3'}$), 5.66 (dd, 1 H, J = 3.0 Hz, 1.8 Hz, $H_{2'}$), 5.46 (d, 1 H, J = 1.8 Hz, $H_{1'}$), 5.14 (s, 1 H, benzylidene CH), 4.71 (s, 1 H, H_1), 4.68 (ddd, J = 9.9 Hz, 4.8 Hz, 1.8 Hz, $H_{5'}$), 4.66 (dd, 1 H, J = 12.0 Hz, 4.8 Hz, $H_{6'}$), 4.55 (dd, 1 H, J = 9.9 Hz, 3.9 Hz, H_3), 4.45 (dd, 1 H, J = 12.0 Hz, 1.8 Hz, $H_{6'}$), 4.13 (t, 1 H, J = 9.9 Hz, H_4), 4.08 (dd, 1 H, J = 10.2 Hz, 5.4 Hz, H_6), 3.78 (ddd, 1 H, J = 10.2 Hz, 9.9 Hz, 5.4 Hz, H_5), 3.63 (m, 2 H, -O-C<u>H</u>$_2$-C<u>H</u>$_2$-N-), 3.51 (t, 1 H, J = 10.2 Hz, H_6), 3.45 (m, 1 H, -O-CH_2-C<u>H</u>$_2$-N-), 3.24 (m, 1 H, -O-C<u>H</u>$_2$-CH_2-N-), 1.91, 1.63, 1.59 & 1.52 (each s, 12 H, COC<u>H</u>$_3$)

^{13}C-NMR (C_6D_6 – 125 MHz) – δ 170.2, 169.6, 169.5 & 169.2 (each s', $\underline{C}OCH_3$), 167.9 (s', phthalimido $\underline{C}$=O), 166.0 (s', benzoyl $\underline{C}$=O), 138.0 (s', benzylidene ring carbon), 133.8 (d', phthalimido –CH-$\underline{C}$H-$\underline{C}$H-CH-), 133.4 (d', benzoyl para $\underline{C}$H), 132.3 (s', benzoyl ring carbon), 130.4 (s', 2 phthalimido ring carbons), 130.4 (d', benzoyl ortho $\underline{C}$H), 128.8 (d', benzoyl meta $\underline{C}$H), 128.7 (d', benzylidene para $\underline{C}$H), 128.5 (d', benzylidene meta $\underline{C}$H), 126.6 (d', benzylidene ortho $\underline{C}$H), 123.2 (d', phthalimido –$\underline{C}$H-CH-CH-$\underline{C}$H-), 101.2 (d', benzylidene $\underline{C}$H), 99.1 (d', $C_{1'}$), 98.6 (d', C_1), 79.3 (d', C_4), 72.1 (d', C_2), 71.8 (d', C_3), 70.2 (d', $C_{2'}$), 70.1 (d', $C_{5'}$), 69.4 (d', $C_{3'}$), 68.6 (t', C_6), 66.4 (d', $C_{4'}$), 64.4 (t', -O-$\underline{C}H_2$-CH_2-N-), 64.3 (d', C_5), 62.6 (t', $C_{6'}$), 37.2 (t', -O-CH_2-$\underline{C}H_2$-N-), 20.5, 20.2 & 20.1 (each q', CO$\underline{C}H_3$)

HR-ESMS for $C_{44}H_{45}NO_{18}Na$ ($M + Na^+$) – Calculated 898.2534; Found 898.2537

2-Phthalimidoethyl 2-$\underline{O}$-benzoyl-2,3,4,6-tetra-$\underline{O}$-acetyl-α-D-mannopyranosyl-(1→3)-α-D-mannopyranoside (**42**)



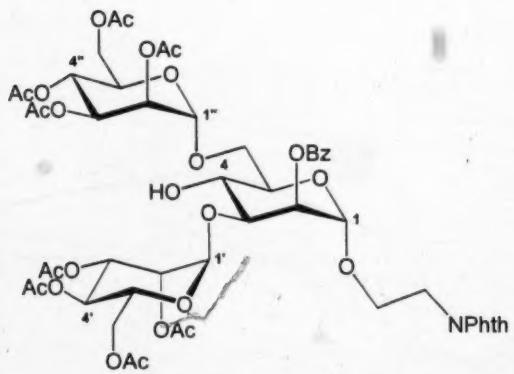
The disaccharide **41** (2.04 g, 2.33 mmol) was stirred in 80% HOAc (50 mL, aq.) at r.t. The slurry was stirred at 80°C for 4 h. The solution was concentrated then co-evaporated with toluene. Purification through column chromatography – eluent polarity ranging from 1% to 5% methanol in toluene – resulted in the isolation of the desired product (938 mg, 51%, R_f = 0.07 in 1:1 EtOAc : $PhCH_3$).

^{1}H-NMR ($CDCl_3$ – 600 MHz) – δ 8.00 (m, 2 H, benzoyl ortho CH), 7.84 (m, 2 H, phthalimido –C<u>H</u>-CH-CH-C<u>H</u>-), 7.70 (m, 2 H, phthalimido –CH-C<u>H</u>-C<u>H</u>-CH-), 7.25 (m, 1 H, benzoyl para CH), 7.14 (m, 2 H, benzoyl meta C<u>H</u>), 5.28 (m, 1 H, H_2), 5.27 (dd, 1 H, J = 3.6 Hz, 1.8 Hz, $H_{2'}$), 5.19 (dd, 1 H, J = 15.9 Hz, 3.4 Hz, $H_{4'}$), 5.14 (m, 1 H, $H_{1'}$), 5.11 (m, 1 H, $H_{3'}$), 4.97 (m, 1 H, H_1), 4.07 to 4.15 (m, 3 H, H_4, $H_{6'}$ & H_3), 3.94 (m, 2 H, $H_{5'}$ & $H_{6'}$), 3.88 (m, 3 H, -O-C<u>H</u>$_2$-C<u>H</u>$_2$-N-), 3.81 (m, 2 H, 2xH_6), 3.74 (m, 1 H, -O-C<u>H</u>$_2$-CH$_2$-N-), 3.64 (m, 1 H, H_5), 2.68 (broad s, 2 H, C_4 & C_6-O<u>H</u>), 2.08, 2.02 & 1.89 (each s, 3 H, CO<u>C</u>H$_3$)

^{13}C-NMR ($CDCl_3$ – 125 MHz) – δ 170.6, 170.1, 170.0 & 169.6 (each s', <u>C</u>OCH$_3$), 168.1 (s', 2 phthalimido <u>C</u>=O), 165.7 (s', benzoyl <u>C</u>=O), 134.1 (d', phthalimido –CH-<u>C</u>H-<u>C</u>H-CH-), 133.4 (d', benzoyl para <u>C</u>H), 131.9 (s', 2 phthalimido ring carbons), 129.8 (d', benzoyl ortho <u>C</u>H), 129.4 (s', benzoyl ring carbon), 128.5 (d', benzoyl meta <u>C</u>H), 123.4 (d', phthalimido –<u>C</u>H-CH-CH-<u>C</u>H-), 99.5 (d', $C_{1'}$), 97.3 (d', C_1), 77.9 (d', C_3), 72.5 (d', C_5), 71.9 (d', C_2), 69.3 (d', $C_{2'}$), 69.1 (d', $C_{5'}$), 69.1 (d', $C_{3'}$), 67.0 (d', C_4), 65.6 (d', $C_{4'}$), 64.7 (t', -O-<u>C</u>H$_2$-CH$_2$-N-), 62.1 (t', $C_{6'}$), 62.0 (t', C_6), 37.4 (t', -O-CH$_2$-<u>C</u>H$_2$-N-), 20.8, 20.6, 20.6 & 20.6 (each q', CO<u>C</u>H$_3$)

HR-ESMS for $C_{37}H_{41}NO_{18}Na$ ($M + Na^+$) – Calculated 810.2221; Found 810.2223

2-Phthalimidoethyl 2-O-benzoyl-2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl-(1→3)-[2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl-(1→6)]-α-D-mannopyranoside (**34**)



To a chilled solution of **38** (163 mg, 0.42 mmol) dissolved in dry dichloromethane (3 mL), was added a solution of HBr/HOAc (33% HBr in HOAc, ca. 0.5 mL) under chilled conditions. The reaction was stirred at r.t. for 1.5 h. Toluene and chloroform were added to the reaction flask. The reaction was concentrated then co-evaporated with toluene until most of the HBr/HOAc had disappeared. The oil was then diluted with chloroform and washed with $NaHCO_3$ (sat., aq.) and brine. After drying over Na_2SO_4, the solution was concentrated and the oil (181 mg, R_f = 0.47 in 3:1:1 hexane : EtOAc : acetone) obtained was used immediately.

In an inert atmosphere was prepared a slurry containing **42** (205 mg, 0.26 mmol), $HgCN_2$ (81 mg, 0.3 mmol) and $HgBr_2$ (114 mg, 0.32 mmol) in dry acetonitrile (2 mL). **35** (181 mg, 0.44 mmol) was added slowly to the slurry at r.t. The syringe was rinsed with dry acetonitrile (3 x 1 mL). The reaction was stirred at r.t. for 2.5 h. The reaction was diluted with acetonitrile then concentrated. The residue was dissolved in $CHCl_3$ then washed with 10% KI (aq.), H_2O and brine. After drying over Na_2SO_4, the solution was concentrated and purified by column

chromatography. Eluent polarity ranging from 7:1.5:1.5 to 6:2:2 hexane : EtOAc : acetone was used. The desired trisaccharide was isolated as a solid (99 mg, 34%, R_f = 0.16 in 3:1:1 hexane : EtOAc : acetone + 1% Et_3N).

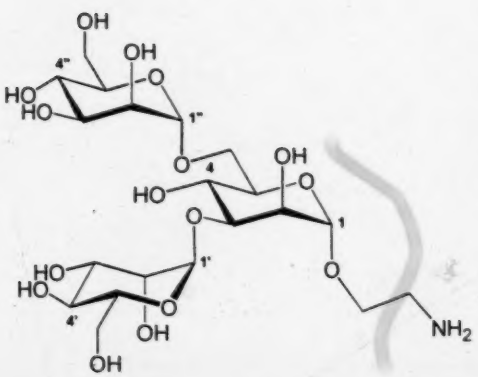
^{1}H-NMR ($CDCl_3$ – 600 MHz) – δ 8.01 (m, 2 H, benzoyl ortho CH), 7.85 (dd, 2 H, J = 5.4 Hz, 3.0 Hz, phthalimido –C<u>H</u>-CH-CH-C<u>H</u>-), 7.70 (dd, 2 H, J = 5.4 Hz, 3.0 Hz, phthalimido –CH-C<u>H</u>-C<u>H</u>-CH-), 7.55 (t, 1 H, J = 7.5 Hz, benzoyl para CH), 7.47 (t, 2 H, J = 7.8 Hz, benzoyl meta CH), 5.35 (dd, 1 H, J = 10.2 Hz, 3.0 Hz, $H_{3''}$), 5.32 (m, 1 H, H_2), 5.29 (dd, 1 H, J = 3.0 Hz, 1.8 Hz, $H_{2''}$), 5.27 (dd, 1 H, J = 3.3 Hz, 1.8 Hz, $H_{2'}$), 5.26 (t, 1 H, J = 10.2 Hz, $H_{4''}$), 5.20 (t, 1 H, J = 9.9 Hz, $H_{4'}$), 5.14 (dd, 1 H, J = 9.9 Hz, 3.3 Hz, $H_{3'}$), 5.12 (d, 1 H, J = 1.8 Hz, $H_{1'}$), 4.96 (d, 1 H, J = 1.8 Hz, H_1), 4.87 (d, 1 H, J = 1.8 Hz, $H_{1''}$), 4.22 (dd, 1 H, J = 12.0 Hz, 5.4 Hz, $H_{6''}$), 4.04 to 4.13 (m, 5 H, $H_{6''}$, -O-C<u>H</u>$_2$-CH$_2$-N-, $H_{5''}$, H_3 & H_4), 3.88 to 3.97 (m, 6 H, -O-C<u>H</u>$_2$-C<u>H</u>$_2$-N-, $H_{5'}$, H_6 & $H_{6'}$), 3.82 (ddd, 1 H, J = 9.8 Hz, 5.3 Hz, 1.7 Hz, H_5), 3.74 (m, 2 H, $H_{6'}$ & H_6), 2.11, 2.09, 2.03, 2.02, 2.01, 1.91, 1.90 & 1.89 (each s, 3 H, COC<u>H</u>$_3$)

^{13}C-NMR ($CDCl_3$ – 125 MHz) – δ 170.7, 170.6, 170.0, 169.8, 169.8, 169.6 & 169.6 (each s', <u>C</u>OCH$_3$), 168.1 (s', 2 phthalimido <u>C</u>=O), 165.7 (s', benzoyl <u>C</u>=O), 134.0 (d', phthalimido –CH-<u>C</u>H-<u>C</u>H-CH-), 133.4 (d', benzoyl para <u>C</u>H), 132.1 (s', 2 phthalimido ring carbons), 129.8 (d', benzoyl ortho <u>C</u>H), 129.4 (s', benzoyl ring carbon), 128.7 (d', benzoyl meta <u>C</u>H), 123.4 (d', phthalimido –<u>C</u>H-CH-CH-<u>C</u>H-), 99.6 (d', $C_{1'}$), 97.4 (d', $C_{1''}$), 97.3 (d', C_1), 78.5 (d', C_4), 71.7 (d', C_2), 71.4 (d', C_5), 69.5 (d', $C_{2'}$ & $C_{2''}$), 69.4 (d', $C_{5'}$), 69.3 (d', $C_{3'}$), 69.0 (d', $C_{3''}$), 68.5 (d', $C_{5''}$), 66.7 (d', C_3), 66.3 (d', $C_{4''}$), 66.2 (t', C_6), 65.6 (d', $C_{4'}$), 64.7 (t', $C_{6'}$), 62.6 (t', $C_{6''}$), 62.1

(t', -O-$\underline{C}H_2$-CH_2-N-), 37.4 (t', -O-CH_2-$\underline{C}H_2$-N-), 20.8, 20.8, 20.7, 20.6, 20.6, 20.6 & 20.6 (each q', $CO\underline{C}H_3$)

HR-ESMS for $C_{51}H_{59}NO_{27}Na$ ($M + Na^+$) – Calculated 1140.3167; Found 1140.3160

2-Aminoethanol α-D-mannopyranosyl-(1→3)-[α-D-mannopyranosyl-(1→6)]-α-D-mannopyranoside (**2**)



The protected trisaccharide **34** (99 mg, 0.1 mmol) was dissolved in dry CH_3OH (2 mL) at r.t. 0.5 M $NaOCH_3$ in CH_3OH (118 µL, 0.06 mmol) was added to the solution at r.t.; the pH was 8. The reaction was stirred at r.t. for 24 h. The reaction was neutralized with Amberlite IR-120 (H^+). Once the pH was < 5, the solution was filtered over Celite then concentrated. A film (63 mg, R_f = 0.22 in 4:1 CH_3CN : H_2O) was isolated and used without further purification.

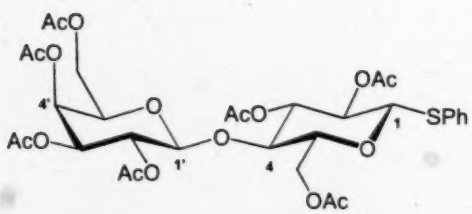
The residue (63 mg, 0.1 mmol) was dissolved in 10 mL 100% EtOH at r.t. H_2NNH_2—H_2O (0.6 mL, 6.6 mmol) was added to the solution at r.t. The reaction was heated at reflux (95°C) for 7 h. The reaction was concentrated and co-evaporated with $PhCH_3$. The crude product was purified using Sephadex G-10 gel filtration and isolated as a film (50 mg, quant. over 2 steps, R_f = 0.32 in 1:2:1 ^{n}BuOH : ^{n}PrOH : 0.1 M HCl).

^{1}H-NMR (D_2O – 500 MHz) – δ 5.11 (d, 1 H, J = 1.5 Hz, $H_{1'}$), 4.91 (d, 1 H, J = 1.5 Hz, $H_{1''}$), 4.87 (d, 1 H, J = 1.5 Hz, H_1), 4.16 (m, 2 H), 4.08 (m, 2 H), 4.04 to 3.87 (m, 10 H), 3.84 to 3.68 (m, 16 H), 3.30 to 3.25 (m, 4 H)

^{13}C-NMR (D_2O – 500 MHz) – δ 103.3 (d', $C_{1'}$), 100.9 (d', C_1), & 100.3 (d', $C_{1''}$), 79.4, 74.2, 73.5, 72.2, 71.4, 71.2, 70.8, 70.7, 70.3, 67.7, 67.5 & 66.2 (each d'), 66.0, 64.3, 61.9 & 61.8 (each t'), 39.9 (t', -O-CH_2-$\underline{C}H_2$-N-)

HR-ESMS for $C_{20}H_{38}NO_{16}$ ($M + H^+$) – Calculated 548.2185; Found 548.2189

Phenyl 2,3,4,6-tetra-<u>O</u>-acetyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-<u>O</u>-acetyl-1-thio-β-D-glucopyranoside (**45**)



The disaccharide **44** (4.9 g, 7.23 mmol) was dissolved in dry DCM (20 mL) at r.t. The solution was chilled in an ice-H_2O bath. Thiophenol (1.1 mL, 10.7 mmol) followed by BF_3—Et_2O (2.7 mL, 21.3 mmol) were added to the reaction. The reaction was stirred at r.t. for 2.5 h. The reaction was quenched with triethylamine then washed with 2 N HCl, H_2O, $NaHCO_3$ (sat., aq.) and brine then dried over Na_2SO_4. The crude oil was purified using column chromatography using 8:1:1 to 6:2:2 hexane : EtOAc : acetone. A white solid was obtained (2.93 g, 56%, R_f = 0.18 in 3:1:1 hexane : EtOAc : acetone).

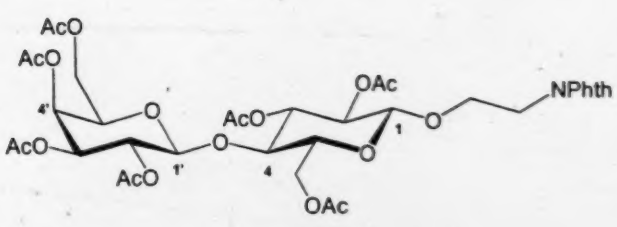
^{1}H-NMR ($CDCl_3$ – 600 MHz) – δ 7.44 (m, 2 H, phenyl o CHs), 7.28 (dd, 3 H, J = 5.1 Hz, 1.5 Hz, phenyl m & p CHs), 5.31 (d, 1 H, J = 3.6 Hz, $H_{4'}$), 5.18 (t, 1 H, J =

10.2 Hz, H_3), 5.08 (dd, 1 H, J = 10.5 Hz, 7.8 Hz, $H_{2'}$), 4.92 (dd, 1 H, J = 10.5 Hz, 3.6 Hz, $H_{3'}$), 4.86 (t, 1 H, J = 10.2 Hz, H_2), 4.64 (d, 1 H, J = 10.2 Hz, H_1), 4.49 (dd, 1 H, J = 12.0 Hz, 1.8 Hz, H_6), 4.43 (d, 1 H, J = 7.8 Hz, $H_{1'}$), 4.08 (dd, 1 H, J = 11.1 Hz, 5.7 Hz, $H_{6'}$), 4.07 (dd, 1 H, J = 12.0 Hz, 6.0 Hz, H_6), 4.02 (dd, 1 H, J = 11.1 Hz, 7.5 Hz, $H_{6'}$), 3.83 (t, 1 H, J = 6.9 Hz, $H_{5'}$), 3.72 (t, 1 H, J = 10.2 Hz, H_4), 3.61 (ddd, 1 H, J = 10.2 Hz, 6.0 Hz, 1.8 Hz, H_5), 2.12, 2.08, 2.06, 2.01, 2.00 & 1.93 (each s, 3 H, $COC\underline{H}_3$)

^{13}C-NMR ($CDCl_3$ – 125 MHz) – δ 170.3, 170.2, 170.1, 170.0, 169.6, 169.5 & 169.0 (each s', $\underline{C}OCH_3$), 133.0 (d', phenyl ortho carbon), 131.7 (s', $\underline{C}$-S), 128.3 & 128.8 (each d', phenyl meta & para carbon), 101.0 (d', $C_{1'}$), 85.5 (d', C_1), 76.7 (d', C_5), 76.1 (d', C_4), 73.8 (d', C_3), 71.0 (d', $C_{3'}$), 70.7 (d', $C_{5'}$), 70.3 (d', C_2), 69.1 (d', $C_{2'}$), 66.6 (d', $C_{4'}$), 62.1 (t', C_6), 60.8 (t', $C_{6'}$), 20.8, 20.7, 20.6, 20.6 & 20.5 (each q', $CO\underline{C}H_3$)

HR-ESMS for $C_{32}H_{40}O_{17}NaS$ ($M + Na^+$) – Calculated 751.1884; Found 751.1887

2-Phthalimidoethyl 2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-O-acetyl-β-D-glucopyranoside (**43**)



A mixture of **24** (636 mg, 3.33 mmol) and **45** (1.05 g, 1.44 mmol) were weighed then dried under vacuum together. The mixture was then stirred in dry dichloromethane (10 mL) at r.t. under inert

atmospheric conditions in the presence of powdered 4Å-molecular sieves (402.4 mg). The solution was stirred for 2 h at r.t. *N*-iodosuccinimide (1.31 g, 5.82 mmol) followed by silver trifluoromethanesulphonic acid (195 mg, 0.87 mmol) were added to the solution. The reaction was stirred at r.t. for 4 h. The reaction was quenched with triethylamine, diluted with chloroform then filtered over Celite. The organic solution was washed with 10% $Na_2S_2O_3$ (aq.) and brine then dried over Na_2SO_4. Purification of the crude oil over column chromatography – eluent polarity ranging from 7:1.5:1.5 to 6:2:2: hexane : EtOAc : acetone – afforded the desired disaccharide as an oil (601 mg, 52%, R_f = 0.52 in 4:3:3 hexane : EtOAc : acetone).

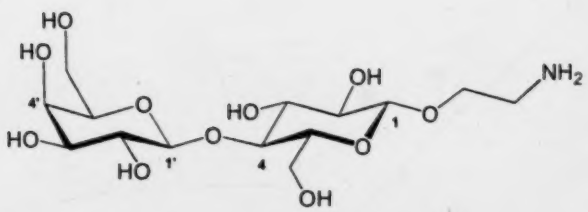
^{1}H-NMR ($CDCl_3$ -- 600 MHz) -- δ 7.81 (m, 2 H, phthalimido -C<u>H</u>-CH-CH-C<u>H</u>-), 7.69 (m, 2 H, phthalimido -CH-C<u>H</u>-C<u>H</u>-CH-), 5.30 (dd, 1 H, J = 3.6 Hz, 0.8 Hz, $H_{4'}$), 5.10 (t, 1 H, J = 9.6 Hz, H_3), 5.05 (dd, 1 H, J = 10.2 Hz, 7.8 Hz, $H_{2'}$), 4.91 (dd, 1 H, J = 10.2 Hz, 3.6 Hz, $H_{3'}$), 4.81 (dd, 1 H, J = 9.6 Hz, 7.8 Hz, H_2), 4.44 (d, 1 H, J = 7.8 Hz, H_1), 4.41 (d, 1 H, J = 7.8 Hz, $H_{1'}$), 4.38 (dd, 1 H, J = 11.7 Hz, 2.1 Hz, H_6), 4.07 (dd, 1 H, J = 11.4 Hz, 6.3 Hz, $H_{6'}$), 4.04 (dd, 1 H, J = 11.4 Hz, 4.8 Hz, $H_{6'}$), 3.96 (m, 2 H, -O-C<u>H</u>$_2$-CH$_2$-N- & H_6), 3.75 to 3.91 (m, 4 H, -O-C<u>H</u>$_2$-C<u>H</u>$_2$-N- & $H_{5'}$), 3.72 (t, 1 H, J = 9.6 Hz, H_4), 3.55 (ddd, 1 H, J = 9.6 Hz, 5.0 Hz, 2.0 Hz, H_5), 2.11, 2.09, 2.03, 2.00, 1.98, 1.93 & 1.83 (each s, 3 H, COC<u>H</u>$_3$)

^{13}C-NMR ($CDCl_3$ -- 125 MHz) -- δ 170.3, 170.3, 170.1, 170.0, 169.7, 169.4 & 169.0 (each s', <u>C</u>OCH$_3$ <u>C</u>=O), 167.9 (s', phthalimido <u>C</u>=O) 134.0 (d', phthalimido -CH-<u>C</u>H-<u>C</u>H-CH-), 132.0 (s', 2 phthalimido ring carbons), 123.3 (d', phthalimido -<u>C</u>H-CH-CH-<u>C</u>H-), 101.0 (d', $C_{1'}$), 100.1 (d', C_1), 76.2 (d', C_4), 72.7 (d', C_3), 72.6

(d', C_5), 71.4 (d', C_2), 71.0 (d', $C_{3'}$), 70.7 (d', $C_{5'}$), 69.1 (d', $C_{2'}$), 66.6 (d', $C_{4'}$), 65.9 (t', -O-$\underline{C}H_2$-CH_2-N-), 61.9 (t', C_6), 60.8 (t', $C_{6'}$), 37.5 (t', -O-CH_2-$\underline{C}H_2$-N-), 20.8, 20.7, 20.6, 20.6, 20.5 & 20.4 (each q', CO$\underline{C}H_3$)

HR-ESMS for $C_{36}H_{43}NO_{20}Na$ ($M + Na^+$) – Calculated 832.2276; Found 832.2276

2-Aminoethanol β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (**3**)



The protected disaccharide **43** (129 mg, 0.16 mmol) was dissolved in dry CH_3OH (3 mL) at r.t. 0.5 M $NaOCH_3$ in CH_3OH (200 µL, 0.1 mmol) was added to the solution at r.t.; the pH was 8. After stirring for 12 h, the reaction was diluted with dry DCM then filtered over Celite and concentrated. A white solid was obtained (68 mg, R_f = 0.59 in 4:1 CH_3CN : H_2O).

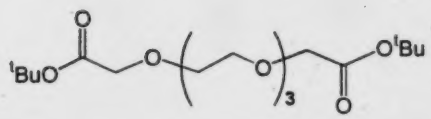
The crude solid was dissolved into 100% EtOH at r.t. H_2NNH_2—H_2O (1.1 mL, 98.2 mmol) was added to the solution. The reaction was heated at reflux (95°C) for 4 h. The reaction was concentrated then co-evaporated with $PhCH_3$ and CH_3OH. The crude residue was purified using Sephadex G-10 gel filtration. A clear film was obtained (55 mg, 90% over 2 steps, R_f = 0.45 in 1:2:1 ^{n}BuOH : ^{n}PrOH : 0.1 M HCl).

^{1}H-NMR (D_2O – 500 MHz) – δ 4.55 (d, 1 H, J = 8.0 Hz, H_1), 4.45 (d, 1 H, J = 8.0 Hz, $H_{1'}$), 4.16 to 4.11 (m, 1 H), 4.01 to 3.94 (m, 4 H), 3.85 to 3.63 (m, 12 H), 3.57 to 3.53 (m, 3 H), 3.40 to 3.37 (mulitplet, 2 H), 3.29 to 3.27 (m, 3 H)

^{13}C-NMR (D_2O – 125 MHz) – δ 103.8 (d', $C_{1'}$), 102.8 (d', C_1), 79.1, 76.2, 75.6, 75.1, 73.5, 73.3, 71.8 & 69.4 (each d'), 66.6, 61.9, 60.8 & 40.2 (each t')

HR-ESMS for $C_{14}H_{28}NO_{11}$ ($M + H^+$) – Calculated 386.1657; Found 386.1658

3,6,9,12-Tetraoxatetradecane-1,14-dioic Acid Di-tert-butyl Ester (**51**)



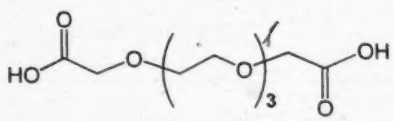
At r.t., NaH (60% dispersion in mineral oil, 0.76 g, 19.0 mmol) was carefully added to a solution of triethylene glycol (**48**, 1.1 g, 7.3 mmol) in 10 mL DMF. The flask was rinsed with 5 mL x 2 DMF to bring any residue of NaH into the reaction mixture. The mixture was stirred at r.t. for a period of 1 h 55 m. Tert-butyl bromoacetate (**50**) dissolved in 5 mL DMF was poured dropwise into the reaction flask chilled at 2°C (ice-H_2O bath). Once the addition was complete, 5 mL DMF was used to rinse the addition funnel. The reaction flask was removed from the ice-H_2O bath and the reaction was stirred at r.t. for 1 h 45 m. The solution was diluted with CH_3CN then co-evaporated with $PhCH_3$ to remove most of DMF; the residue was dissolved into ethyl acetate and the organic solution was then washed with NH_4Cl (sat.), $NaHCO_3$ (sat.) and NaCl (sat.). After drying over Na_2SO_4, the organic solution was concentrated then purified by column chromatography – eluent polarity ranging from 1:1 to 1:4 toluene : ethyl acetate + 1% Et_3N. The desired product was obtained as an oil (1.03 g, 38%, R_f = 0.57 in 1:4 $PhCH_3$: EtOAc).

^{1}H-NMR ($CDCl_3$ – 500 MHz) – δ 3.97 (s, 4 H, -O-C<u>H</u>$_2$CO$_2$^{t}Bu), 3.62 to 3.67 (m, 12 H, -O-C<u>H</u>$_2$-C<u>H</u>$_2$-O-), 1.43 (s, 18 H, $CO_2C(C\underline{H}_3)_3$)

^{13}C-NMR ($CDCl_3$ – 125 MHz) – δ 169.6 (s', -O-CH$_2$<u>C</u>O$_2$^{t}Bu), 81.4 (s', <u>C</u>(CH$_3$)$_3$), 70.5 & 70.7 (each t', -O-<u>C</u>H$_2$-<u>C</u>H$_2$-O-), 69.0 (t', -O-<u>C</u>H$_2$CO$_2$^{t}Bu), 28.1 (q', $CO_2C(\underline{C}H_3)_3$)

HR-ESMS for $C_{18}H_{34}O_8Na$ ($M + Na^+$) – Calculated 401.2146; Found 401.2143

3,6,9,12-Tetraoxatetradecane-1,14-dioic Acid (**46**)



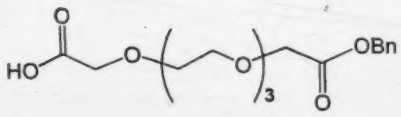
The oily starting material **51** (0.76 g, 2.01 mmol) was dissolved in 3.3 mL DCM at r.t. Trifluoroacetic acid (3.3 mL, 42.8 mmol) was added to the solution at r.t. The reaction was stirred at r.t. for 1 h. The reaction was diluted with $CHCl_3$, concentrated then co-evaporated with $PhCH_3$. The desired product was obtained as an oil in a quantitative yield (0.58 g, R_f = 0.24 in 4:1 CH_3CN : H_2O).

^{1}H-NMR ($CDCl_3$ – 500 MHz) – δ 4.15 (s, 4 H, -O-C<u>H</u>$_2$CO$_2$H), 3.64 to 3.74 (m, 12 H, -O-C<u>H</u>$_2$-C<u>H</u>$_2$-O-)

^{13}C-NMR ($CDCl_3$ – 125 MHz) – δ 173.1 (s', -O-CH$_2$<u>C</u>O$_2$H), 70.3 (t', -O-CH$_2$-CH$_2$-O-), 68.6 (t', -O-<u>C</u>H$_2$CO$_2$H)

HR-ESMS for $C_{10}H_{19}O_8$ ($M + H^+$) – Calculated 267.1074; Found 267.1076

3,6,9,12-Tetraoxatetradecane-1-ic Acid-14-Benzyl Ester (**14**)



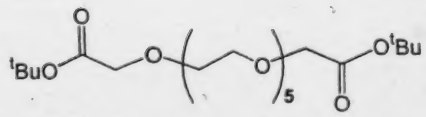
The pre-dried di-acid, **46** (261 mg, 0.98 mmol) was dissolved in CH_3CN (2.5 mL) at r.t. Triethylamine (260 µL, 1.87 mmol), followed by benzyl bromide (120 µL, 1.01 mmol) were added to the solution at r.t. The reaction was stirred for 1 h 40 m at r.t. The reaction was diluted with CH_3CN then concentrated. The residue was dissolved into $CHCl_3$ then washed with 1 N HCl sat. with NaCl and dried over Na_2SO_4. The crude product was purified further with EtOAc : CH_3CN = 9.5 : 0.5 (gravity) followed by CH_3CN : H_2O = 4 : 1 (flash). The product was obtained as an oil (94 mg, 27%, R_f = 0.62 in 4:1 CH_3CN : H_2O).

^{1}H-NMR ($CDCl_3$ – 500 MHz) – δ 7.34 (s, 5 H, benzyl aromatic C<u>H</u>), 5.17 (s, 2 H, benzyl C<u>H</u>$_2$), 4.20 (s, 2 H, -O-C<u>H</u>$_2$CO$_2$Bn), 4.18 (s, 2 H, -O-C<u>H</u>$_2$CO$_2$H), 3.64 to 3.71 (m, 12 H, -O-C<u>H</u>$_2$-C<u>H</u>$_2$-O-)

^{13}C-NMR ($CDCl_3$ – 125 MHz) – δ 170.8 (s', -O-CH$_2$<u>C</u>O$_2$H & -O-CH$_2$-<u>C</u>O$_2$Bn), 135.2 (s', benzyl ring carbon), 128.3 to 128.6 (d', benzyl <u>C</u>H), 70.6 to 70.0 (t', -O-<u>C</u>H$_2$-<u>C</u>H$_2$-O-), 68.3 (t', -O-<u>C</u>H$_2$CO$_2$H & -O-<u>C</u>H$_2$-CO$_2$Bn), 66.8 (t', benzyl <u>C</u>H$_2$)

HR-ESMS for $C_{17}H_{24}O_8Na$ ($M + Na^+$) – Calculated 379.1363; Found 379.1367

3,6,9,12,15,18-Hexaoxaeicosane-1,20-dioic Acid Di-tert-butyl Ester (**52**)



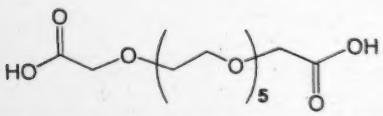
A solution of pentaethylene glycol (**49**, 1.62 g, 6.8 mmol) in 20 mL toluene was prepared. Once the solution had been chilled at 3°C, 50% NaOH (20 mL, 0.25 mol) was added. Tetrabutylammonium hydrogensulphate (5.1 g, 15.0 mmol) and tert-butyl bromoacetate (**50**, 4.1 mL, 27.8 mmol) were added to the reaction flask; the mixture was stirred vigorously while chilled at 3°C. After 10 m, the flask was removed from the ice-H_2O bath and allowed to stand at r.t. Once the flask had reached r.t., the reaction was stirred for another 30 m. The reaction mixture was poured into a separatory funnel containing H_2O; the aqueous layer was removed. The organic layer was diluted with ethyl acetate (5 mL) then washed with NH_4Cl (sat.), $NaHCO_3$ (sat.) and NaCl (sat.). The organic layer was dried with Na_2SO_4 then concentrated. Purification using column chromatrography – toluene : ethyl acetate = 1:4 – afforded 1.96 g (61%, R_f = 0.30 in 1:4 $PhCH_3$: EtOAc) the desired product.

^{1}H-NMR ($CDCl_3$ – 500 MHz) – δ 4.00 (s, 4 H, -O-C$\underline{H_2}$CO$_2$^{t}Bu), 3.63 to 3.70 (m, 20 H, -O-C$\underline{H_2}$-C$\underline{H_2}$-O-), 1.46 (s, 18 H, $CO_2C(C\underline{H_3})_3$)

^{13}C-NMR ($CDCl_3$ – 125 MHz) – δ 169.6 (s', -O-CH$_2\underline{C}$O$_2$^{t}Bu), 81.4 (s', $\underline{C}(CH_3)_3$), 70.5, 70.5, 70.6, 70.6, 70.7 (each t', -O-$\underline{C}$H$_2$-$\underline{C}$H$_2$-O-), 69.0 (t', -O-$\underline{C}$H$_2$CO$_2$^{t}Bu), 28.1 (q', $CO_2C(\underline{C}H_3)_3$)

HR-ESMS for $C_{22}H_{42}O_{10}Na$ ($M + Na^+$) – Calculated 489.2670; Found 489.2677

3,6,9,12,15,18-Hexaoxaeicosane-1,20-dioic Acid (**47**)



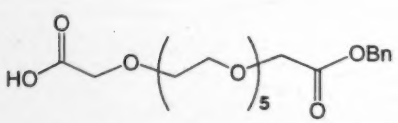
A solution of **52** (1.66 g, 3.56 mmol) in dry dichloromethane (4 mL) was prepared. At r.t., trifluoroacetic acid (5.6 mL) was added to the solution. The reaction was stirred at r.t. for a period of 1.5 h. After TLC confirmation, the reaction was diluted with $CHCl_3$ then concentrated. The residue was co-evaporated with toluene then $CHCl_3$ before drying under vacuum overnight. The desired di-acid was obtained in a quantitative yield (1.90 g, R_f = 0.04 in 4:1 CH_3CN : H_2O).

^{1}H-NMR ($CDCl_3$ – 500 MHz) – δ 4.20 (s, 4 H, -O-C$\underline{H}_2$CO$_2$H), 3.76 to 3.77 (m, 4 H, -O-C$\underline{H}_2$-C$\underline{H}_2$-O-), 3.68 to 3.71 (m, 16 H, -O-C$\underline{H}_2$-C$\underline{H}_2$-O-)

^{13}C-NMR ($CDCl_3$ – 125 MHz) – δ 174.1 (s', -O-CH$_2$C$\underline{O}_2$H), 70.2, 70.2, 70.3 & 70.3 (each t', -O-C$\underline{H}_2$-C$\underline{H}_2$-O-), 68.6 (t', -O-C$\underline{H}_2$CO$_2$H)

HR-ESMS for $C_{14}H_{26}O_{10}Na$ ($M + Na^+$) – Calculated 377.1418; Found 377.1412

3,6,9,12,15,18-Hexaoxaeicosane-1-ic Acid-20-Benzyl Ester (**15**)



The pre-dried di-acid, **47** (109 mg, 308 mmol) was dissolved in 1.5 mL CH_3CN at r.t. DBU (46 µL, 308.2 mmol) dissolved in 0.1 mL CH_3CN was added to the solution at r.t. Benzyl bromide (33 mL, 277.5 mmol) dissolved in 0.1 mL CH_3CN was added to the reaction at r.t. After stirring for 19 h 30 m, the reaction was diluted with $CHCl_3$ then concentrated. The residue was dissolved in $CHCl_3$ then washed with

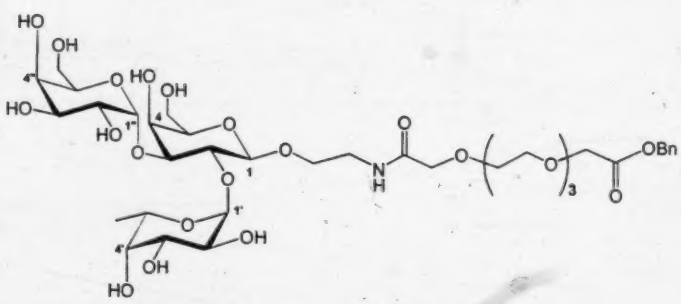
1 N HCl sat. with NaCl; dried over Na_2SO_4. The crude residue was purified over silica using EtOAc : CH_3CN = 4:1 (flash) then CH_3CN : H_2O = 4:1 (flash). The final product was obtained as an oil (39 mg, 28%, R_f = 0.55 in 4:1 CH_3CN : H_2O).

^{1}H-NMR ($CDCl_3$ – 500 MHz) – δ 7.36 (s, 5 H, benzyl aromatic C<u>H</u>), 5.20 (s, 2 H, benzyl C<u>H</u>$_2$), 4.20 & 4.09 (s, 4 H, -O-C<u>H</u>$_2$-CO_2H & -O-C<u>H</u>$_2$-CO_2Bn), 3.68 to 3.63 (m, 20 H, -O-C<u>H</u>$_2$-C<u>H</u>$_2$-O-)

^{13}C-NMR ($CDCl_3$ – 125 MHz) – δ 173.0 (s', -O-CH_2-<u>C</u>O_2H or -O-CH_2-<u>C</u>O_2Bn), 170.9 (s', -O-CH_2-<u>C</u>O_2H or -O-CH_2-<u>C</u>O_2Bn), 135.2 (s', benzyl ring carbon), 128.7, 128.5 & 128.3 (each d', benzyl <u>C</u>H), 70.6 & 69.8 to 69.5 (each t', -O-CH_2-<u>C</u>O_2H or -O-CH_2-<u>C</u>O_2Bn & -O-<u>C</u>H_2-<u>C</u>H_2-O-), 68.2 (each t', -O-CH_2-<u>C</u>O_2H or -O-CH_2-<u>C</u>O_2Bn), 66.7 (t', benzyl <u>C</u>H_2)

HR-ESMS for $C_{21}H_{32}O_{10}Na$ ($M + Na^+$) – Calculated 467.1888; Found 467.1887

7,10,13,16-Tetraoxatetradecane-18-acid-5-amide-1-{α-L-fucopyranosyl-(1→2)-[α-D-galactopyranosyl-(1→3)]-β-D-galactopyranoside}-18-Benzyl Ester (**53**)



The primary amine **1** (16 mg, 30 μmol), and the carboxylic acid, **14** (14 mg, 40 μmol), were dried under vacuum at r.t. for 13 h. The carboxylic acid was dissolved in 100 μL DMF and 100 μL DCM at r.t. The solution was chilled to 1°C (ice-H_2O bath). DCC (9 mg, 45.6 μmol) was

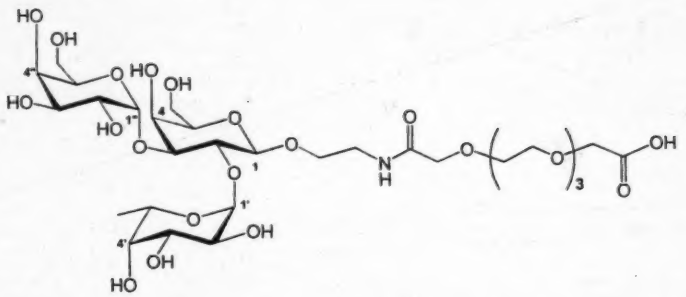
added to the solution in portions using 250 µL DCM. HOBT (6 mg, 45 µmol) was added to the reaction in portions using 300 µL DMF. The primary amine was added to the reaction in portions using 500 µL DMF; 500 µL DCM was added to the reaction. The reaction mixture was stirred at r.t. 10 h 45 m. The reaction was diluted with CH_3CN then concentrated. The residue was co-evaporated with CH_3CN and $PhCH_3$. The residue was triturated with DCM and EtOAc. The crude product was purified further using Sephadex G-10 gel filtration. The coupled product was obtained in 57% yield (15 mg, R_f = 0.52 in 4:1 CH_3CN : H_2O).

^{1}H-NMR (D_2O – 500 MHz) – δ 7.45 (s, 5 H, benzyl aromatic C<u>H</u>), 5.25 (s, 4 H, benzyl C<u>H</u>$_2$, $H_{1''}$ & $H_{1'}$), 4.56 (m, 1 H, H_1), 4.36 to 4.21 (m, 5 H), 4.09 to 3.86 (m, 10 H), 3.86 to 3.66 (m, 28 H), 3.58 to 3.42 (m, 3 H), 1.18 (broad singlet, 3 H)

^{13}C-NMR (D_2O – 125 MHz) – δ 173.4 & 173.1 (each s', -O-CH_2-<u>C</u>O_2Bn & -O-CH_2-<u>C</u>ONHCH_2-), 136.0 (s', benzyl ring carbon), 129.7, 129.6 & 129.2 (each d', benzyl CH), 102.3 (d', C_1), 99.5 (d', $C_{1''}$), 93.9 (d', $C_{1'}$), 77.1, 75.5, 73.6, 72.7 & 72.0 (each d'), 71.1, 71.1, 71.0, 70.6, 70.4, 70.4, 70.3 & 70.2 (each t'), 70.2, 68.9 (each d'), 68.8 & 68.8 (each t'), 68.7 (d'), 68.6, 68.3, 68.1 & 68.1 (each t'), 67.6 & 64.3 (each d'), 62.1 & 61.8 (each t'), 53.2 (d'), 40.0 (t'), 16.1 (q')

HR-ESMS for $C_{37}H_{59}NO_{22}Na$ ($M + Na^+$) – Calculated 892.3421; Found 892.3423

7,10,13,16-Tetraoxatetradecane-18-acid-5-amide-1-{α-L-fucopyranosyl-(1→2)-[α-D-galactopyranosyl-(1→3)]-β-D-galactopyranoside} (**16**)



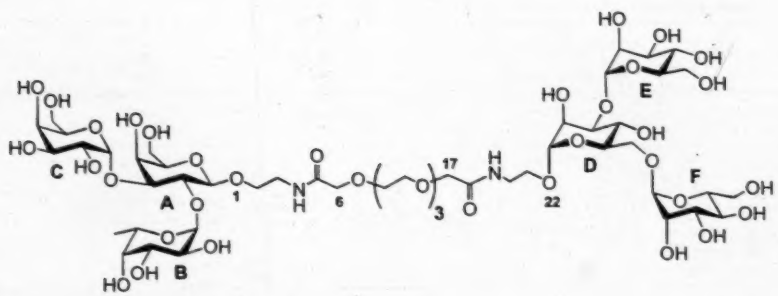
The protected benzyl ester **53** (29 mg, 34 µmol), was dissolved in 2 mL CH_3OH at r.t. $Pd(OH)_2/C$ (49 mg) was added to the solution at r.t. A balloon of H_2 gas was used to remove air from the reaction flask. The reaction was placed under balloon pressure of H_2 gas for 48 h. The reaction was removed from H_2 gas then filtered over Celite before the solution was concentrated. The crude product was further purified using Sephadex G-10 gel filtration and isolated (15 mg, 55%, R_f = 0.04 in 4:1 CH_3CN : H_2O).

^{1}H-NMR (D_2O – 500 MHz) – δ 5.26 (d, 1 H, J = 2.5 Hz, $H_{1''}$), 5.24 (d, 1 H, J = 3.5 Hz, $H_{1'}$), 4.58 (d, 1 H, J = 8.0 Hz, H_1), 4.38 to 4.34 (m, 1 H), 4.28 to 4.21 (m, 3 H), 4.09 (s, 2 H), 4.04 to 3.69 (m, 37 H), 3.52 to 3.49 (m, 4 H), 1.18 (d, 3 H, J = 6.5 Hz, $H_{6'}$)

^{13}C-NMR (D_2O – 125 MHz) – δ 173.5 (s', -O-CH_2-$\underline{C}O_2H$ & -O-CH_2-$\underline{C}$ONH-CH_2-), 102.3 (d', C_1), 99.5 (d', $C_{1''}$), 93.9 (d', $C_{1'}$), 77.1, 75.5, 73.5, 72.7 & 72.0 (each d'), 71.1 & 71.0 (each t'), 70.6 (d'), 70.6, 70.5, 70.4, 70.4, 70.3 & 70.2 (each t'), 70.1, 68.9 & 68.7 (each d'), 68.6, 68.5 & 68.3 (each t'), 67.6 & 64.2 (each d'), 62.1, 61.8 & 40.0 (each t'), 16.1 (q', $C_{6'}$)

HR-ESMS for $C_{30}H_{53}N_1O_{22}Na$ ($M + Na^+$) – Calculated 802.2957; Found 802.2952

7,10,13,16-Tetraoxatetradecane-5,18-diamide-1-{α-L-fucopyranosyl-(1→2)-[α-D-galactopyranosyl-(1→3)]-β-D-galactopyranoside}-22-{α-D-mannopyranosyl-(1→3)-[α-D-mannopyranosyl-(1→6)]-α-D-mannopyranoside} (**10**)



The primary carboxylic acid **16** (7 mg, 9 μmol), and primary amine, **2** (4 mg, 8 μmol), were dried under vacuum at r.t. for 7 h. The carboxylic acid was dissolved in 100 μL DCM and 100 μL DMF at r.t.; the solution was chilled to 1°C (ice-H_2O bath). DCC (3 mg, 14 μmol) was added to the solution in portions using 250 μL DCM. HOBT (2 mg, 15 μmol) was added to the reaction in portions using 300 μL DMF. The primary amine was added to the reaction in portions using 500 μL DMF. The reaction was stirred at r.t. 22 h 45 m. The reaction was concentrated then co-evaporated with $PhCH_3$. The residue was triturated with DCM and EtOAc. The crude product was purified further using Sephadex G-15 gel filtration. The coupled product was obtained in 95% yield (10 mg, R_f = 0.32 in 2:1 ^{i}PrOH : 1 M NH_4OAc).

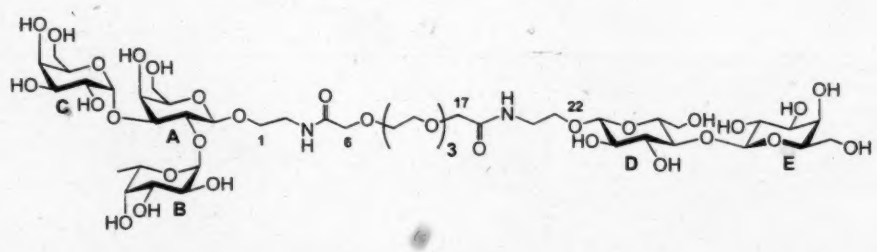
^{1}H-NMR (D_2O – 500 MHz) – δ 5.26 (d, 1 H, J = 2.0 Hz, Ring **C**-H_1), 5.24 (d, 1 H, J = 3.5 Hz, Ring **B**-H_1), 5.09 (d, 1 H, J = 1.5 Hz, Ring **E**-H_1), 4.89 (d, 1 H, J = 1.5 Hz, Ring **F**-H_1), 4.83 (d, 1 H, J = 1.5 Hz, Ring **D**-H_1), 4.58 (d, 1 H, J = 8.0 Hz, Ring **A**-H_1), 4.36 to 4.33 (m, 2 H), 4.27 to 4.21 (m, 4 H), 4.10 to 4.06 (m, 7 H),

4.01 to 3.94 (m, 6 H), 3.90 to 3.86 (m, 9 H), 3.86 to 3.61 (m, 45 H), 3.55 to 3.46 (m, 6 H), 1.18 (d, 3 H, J = 6.5 Hz, $H_{6'}$)

^{13}C-NMR (D_2O – 125 MHz) – δ 173.5 (s', -CH_2-CONH-CH_2-), 103.3 (d', Ring **E**-C_1), 102.4 (d', Ring **A**-C_1), 100.7 (d', Ring **D**-C_1), 100.3 (d', Ring **F**-C_1), 99.6 (d', Ring **C**-C_1), 94.0 (d', Ring **B**-C_1), 79.6, 77.1, 75.6, 74.2, 73.7, 73.6, 72.8, 72.1, 72.1 & 71.5 (each d'), 71.4 (t'), 71.3 (d'), 71.3, 71.2 & 71.1 (each t'), 71.0, 70.9 & 70.7 (each d'), 70.6, 70.5, 70.5, 70.4, 70.4 & 70.4 (each t'), 70.2 (d'), 69.8 (t'), 69.0 & 68.7 (each d'), 68.7 & 68.4 (each t'), 67.7 & 67.6 (each d'), 66.8 (t'), 66.4 (t'), 66.2 (d'), 64.3 (t'), 62.2, 61.9, 40.1, 39.4 (t'), 16.2 (q', $C_{6'}$)

LR-ESMS for $C_{50}H_{88}N_2O_{37}Na$ ($M + Na^+$) – Calculated 1331.5; Found 1331.5

7,10,13,16-Tetraoxatetradecane-5,18-diamide-1-{α-L-fucopyranosyl-(1→2)-[α-D-galactopyranosyl-(1→3)]-β-D-galactopyranoside}-22-{β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside} (**11**)



The primary carboxylic acid **16** (6 mg, 8 μmol), and primary amine **3** (3 mg, 8 μmol), were dried under vacuum at r.t. for 7 h. The carboxylic acid was dissolved in 100 μL DCM and 100 μL DMF at r.t.; the solution was chilled to 1°C (ice-H_2O bath). DCC (3 mg, 13 μmol) was added to the solution in portions using 250 μL DCM. HOBT (2 mg, 13 μmol) was

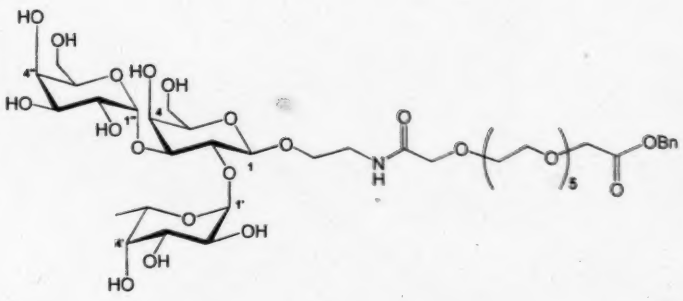
added to the reaction in portions using 300 µL DMF. The primary amine was added to the reaction in portions using 500 µL DMF. The reaction was stirred at r.t. 22 h 45 m. The reaction was concentrated then co-evaporated with $PhCH_3$. The residue was triturated with DCM and EtOAc. The crude product was purified further using Sephadex G-15 gel filtration. The coupled product was obtained in 64% yield (6 mg, R_f = 0.34 in 2:1 ^{i}PrOH : 1 M NH_4OAc).

^{1}H-NMR (D_2O – 600 MHz) – δ 5.26 (d, 1 H, J = 1.8 Hz, Ring **C**-H_1), 5.23 (s, 1 H, J = 3.6 Hz, Ring **B**-H_1), 4.57 (d, 1 H, J = 7.8 Hz, Ring **A**-H_1), 4.48 (d, 1 H, J = 7.8 Hz, Ring **D**-H_1), 4.43 (d, 1 H, J = 7.8 Hz, Ring **E**-H_1), 4.36 to 4.32 (m, 1 H), 4.26 to 4.20 (m, 4 H), 4.09 (s, 3 H), 4.00 to 3.30 (m, 62 H), 1.17 (d, 3 H, J = 6.0 Hz, $H_{6'}$)

^{13}C-NMR (D_2O – 125 MHz) – δ 173.6 (s', -CH_2-$\underline{C}$ONH-CH_2-), 103.8 (d', Ring **E**-C_1), 103.2 (d', Ring **D**-C_1), 102.4 (d', Ring **A**-C_1), 99.6 (d', Ring **C**-C_1), 94.0 (d', Ring **B**-C_1), 79.3, 77.1, 76.3, 75.7, 75.6, 75.6, 75.6, 75.6, 75.2, 73.6, 73.6, 73.4, 72.8, 72.0 & 71.9 (each d'), 71.4, 71.2, 71.2, 71.1 (each t'), 70.7 (d'), 70.5, 70.4, 70.4, 70.4 & 70.3 (each t'), 70.3 (d'), 69.8 (t'), 69.4 (d'), 69.3 (d'), 69.0 (t'), 68.7 (d'), 68.7, 68.5 & 68.4 (each t'), 67.7 (t'), 64.3 (t'), 62.2, 61.9, 61.8, 61.8, 61.0, 40.1 & 39.7 (each t'), 16.2 (q', $C_{6'}$)

LR-ESMS for $C_{44}H_{78}N_2O_{32}Na$ ($M + Na^+$) – Calculated 1169.4; Found 1169.4

7,10,13,16,19,22-Hexaoxaeicosane-24-acid-5-amide-1-{α-L-fucopyranosyl-(1→2)-[α-D-galactopyranosyl-(1→3)]-β-D-galactopyranoside}-24-Benzyl Ester (**54**)



The primary amine **1** (16 mg, 31 μmol), and the carboxylic acid **15** (18 mg, 41 μmol), were dried under vacuum at r.t. for 13 h. The carboxylic acid was dissolved in 100 μL DMF and 100 μL DCM at r.t. The solution was chilled to 1°C (ice-H_2O bath). DCC (10 mg, 46 μmol) was added to the solution in portions using 250 μL DCM. HOBT (6 mg, 45 μmol) was added to the reaction in portions using 300 μL DMF. The primary amine was added to the reaction in portions using 500 μL DMF; 500 μL DCM was added to the reaction. The reaction was stirred at r.t. 10 h 45 m. The reaction was diluted with CH_3CN then concentrated. The residue was co-evaporated with CH_3CN and $PhCH_3$. The residue was triturated with DCM and EtOAc. The crude product was purified further using Sephadex G-10 gel filtration. The coupled product was obtained in 55% yield (16 mg, R_f = 0.55 in 4:1 CH_3CN : H_2O).

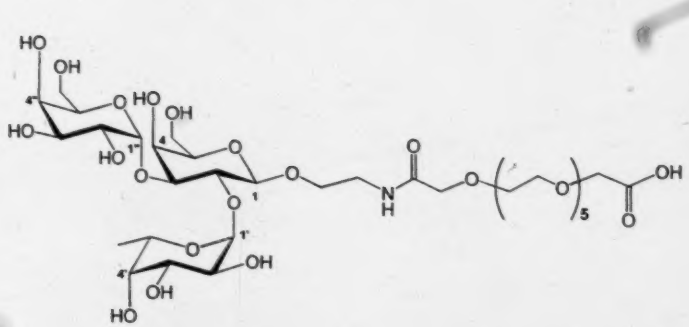
^{1}H-NMR (D_2O – 500 MHz) – δ 7.45 (s, 5 H, benzyl C<u>H</u>), 5.26 (s, 3 H, benzyl CH_2 & $H_{1''}$), 5.24 (d, 1 H, J = 3.5 Hz, $H_{1'}$), 4.57 (d, 1 H, J = 8.0 Hz, H_1), 4.36 to 4.21

(m, 6 H), 4.01 to 3.65 (m, 44 H), 3.52 to 3.46 (m, 4 H), 1.18 (d, 3 H, J = 6.5 Hz, $H_{6'}$)

^{13}C-NMR (D_2O – 125 MHz) – δ 173.4 & 173.0 (s', -O-CH_2-CONH-CH_2 & -O-CH_2-CO_2Bn), 136.0 (s', benzyl ring carbon), 129.7, 129.6 & 129.2 (d', benzyl CH), 102.3 (d', C_1), 99.5 (d', $C_{1''}$), 93.9 (d', $C_{1'}$), 77.1, 75.5, 73.6, 72.7, 72.0 (each d'), 71.1 & 71.0 (each t'), 70.6 (d'), 70.4, 70.3 & 70.3 (each t'), 70.1 & 68.9 (each d'), 68.8 (t'), 68.7 (d'), 68.6, 68.3 & 68.1 (each t'), 67.6 & 64.2 (each d'), 62.1, 61.8 & 40.0 (each t'), 16.1 (q')

HR-ESMS for $C_{41}H_{67}N_1O_{24}Na$ ($M + Na^+$) – Calculated 980.3945; Found 980.3944

7,10,13,16,19,22-Hexaoxaeicosane-24-acid-5-amide-1-{α-L-fucopyranosyl-(1→2)-[α-D-galactopyranosyl-(1→3)]-β-D-galactopyranoside} (**17**)



The protected benzyl ester **54** (31 mg, 32 μmol), was dissolved in 2 mL CH_3OH at r.t. $Pd(OH)_2/C$ (52 mg) was added to the solution at r.t. A balloon of H_2 gas was used to remove air from the reaction flask. The reaction was placed under balloon pressure of H_2 gas for 19 h 45 m. The reaction was removed from H_2 gas then filtered over Celite before the solution was concentrated. The crude product was further purified using

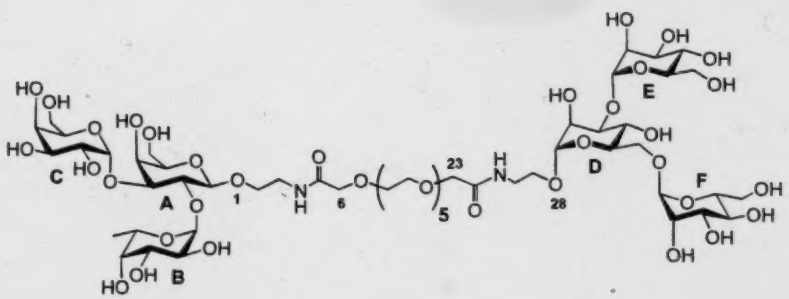
Sephadex G-10 gel filtration and isolated (22 mg, 79%, R_f = 0.05 in 4:1 CH_3CN : H_2O).

^{1}H-NMR (D_2O – 500 MHz) – δ 5.26 (d, 1 H, J = 2.0 Hz, $H_{1''}$), 5.24 (d, 1 H, J = 3.5 Hz, $H_{1'}$), 4.58 (d, 1 H, J = 7.5 Hz, H_1), 4.38 to 4.34 (m, 1 H), 4.29 to 4.21 (m, 3 H), 4.10 to 4.08 (m, 6 H), 4.04 to 3.95 (m, 3 H), 3.90 to 3.69 (m, 39 H), 3.52 to 3.49 (m, 3 H), 1.18 (d, 3 H, J = 6.5 Hz, $H_{6'}$)

^{13}C-NMR (D_2O – 125 MHz) – δ 176.9 & 173.5 (each s', -O-CH_2-$\underline{C}O_2H$ & -O-CH_2-$\underline{C}$ONH-CH_2-), 102.3 (d', C_1), 99.5 (d', $C_{1''}$), 93.9 (d', $C_{1'}$), 77.1, 75.5, 73.5, 72.7 & 72.0 (each d'), 71.1 & 71.0 (each t'), 70.6 (d'), 70.6, 70.4, 70.4 & 70.3 (each t'), 70.1 (d'), 69.5 & 69.4 (each t'), 68.9 & 68.7 (each d'), 68.6 & 68.3 (each t'), 67.6 & 64.2 (each d'), 62.1, 61.8 & 40.0 (each t'), 16.1 (q')

HR-ESMS for $C_{34}H_{61}N_1O_{24}Na$ ($M + Na^+$) – Calculated 890.3481; Found 890.3484

7,10,13,16,19,22-Hexaoxaeicosane-5,24-diamide-1-{α-L-fucopyranosyl-(1→2)-[α-D-galactopyranosyl-(1→3)]-β-D-galactopyranoside}-28-{α-D-mannopyranosyl-(1→3)-[α-D-mannopyranosyl-(1→6)]-α-D-mannopyranoside} (**12**)



The primary carboxylic acid **17** (13 mg, 15 μmol), and primary amine **2** (6 mg, 11 μmol), were dried under vacuum at r.t. for 7 h. The carboxylic acid was dissolved in 50 μL DCM and 250 μL DMF at r.t.; the solution

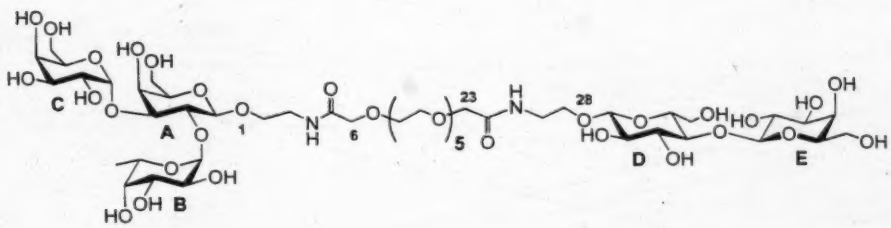
was chilled to 2°C (ice-H_2O bath). DCC (5 mg, 25 μmol) was added to the solution in portions using 250 μL DCM. HOBT (3 mg, 23 μmol) was added to the reaction in portions using 300 μL DMF. The primary amine was added to the reaction in portions using 450 μL DMF; 500 μL DCM was added to the reaction. The reaction was stirred at r.t. 15 h 45 m. The reaction was diluted with CH_3CN then co-evaporated with CH_3CN & $PhCH_3$. The residue was triturated with EtOAc. The crude product was purified further using Sephadex G-15 gel filtration. The coupled product was obtained in 84% yield (13 mg, R_f = 0.32 in 2:1 ^{i}PrOH : 1 M NH_4OAc).

^{1}H-NMR (D_2O – 500 MHz) – δ 5.26 (d, 1 H, J = 1.5 Hz, Ring **C**-H_1), 5.24 (d, 1 H, J = 3.0 Hz, Ring **B**-H_1), 5.09 (d, 1 H, J = 2.0 Hz, Ring **E**-H_1), 4.89 (d, 1 H, J = 1.5 Hz, Ring **F**-H_1), 4.84 (d, 1 H, J = 1.5 Hz, Ring **D**-H_1), 4.58 (d, 1 H, J = 7.5 Hz, Ring **A**-H_1), 4.37 to 4.33 (m, 1 H), 4.28 to 4.22 (m, 3 H), 4.10 to 4.06 (m, 4 H), 4.02 to 3.95 (m, 4 H), 3.90 to 3.85 (m, 6 H), 3.84 to 3.63 (m, 54 H), 3.53 to 3.48 (m, 5 H), 1.18 (d, 3 H, J = 6.5 Hz, $H_{6'}$)

^{13}C-NMR (D_2O – 125 MHz) – δ 173.5 (s', -CH_2-$\underline{C}$ONH-CH_2-), 103.3 (d', Ring **E**-C_1), 102.4 (d', Ring **A**-C_1), 100.7 (d', Ring **D**-C_1), 100.3 (d', Ring **F**-C_1), 99.6 (d', Ring **C**-C_1), 94.0 (d', Ring **B**-C_1), 79.6, 77.1, 75.6, 74.2, 73.6, 73.6, 72.8, 72.1, 72.0, 71.5 (each d'), 71.4 (t'), 71.3 (d'), 71.3, 71.2 & 71.1 (each t'), 71.0, 70.9 & 70.7 (each d'), 70.5, 70.5, 70.4 & 70.4 (each t'), 70.2 (d'), 69.8 (t'), 69.0 & 68.7 (each d'), 68.7 & 68.4 (each t'), 67.7 & 67.6 (each d'), 66.8 (t'), 66.4 (d'), 66.2 (t'), 64.3 (d'), 62.2, 61.9, 40.1 & 39.4 (each t'), 16.2 (q')

LR-ESMS for $C_{54}H_{96}N_2O_{39}Na$ ($M + Na^+$) – Calculated 1419.6; Found 1419.5

7,10,13,16,19,22-Hexaoxaeicosane-5,24-diamide-1-{α-L-fucopyranosyl-(1→2)-[α-D-galactopyranosyl-(1→3)]-β-D-galactopyranoside}-28-{β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside} (**13**)



The primary carboxylic acid **17** (9 mg, 10 μmol), and primary amine **3** (6 mg, 15 μmol), were dried under vacuum at r.t. for 7 h. The carboxylic acid was dissolved in 50 μL DCM and 250 μL DMF at r.t.; the solution was chilled to 2°C (ice-H_2O bath). DCC (3 mg, 16 μmol) was added to the solution in portions using 250 μL DCM. HOBT (2 mg, 16 μmol) was added to the reaction in portions using 300 μL DMF. The primary amine was added to the reaction in portions using 450 μL DMF; 500 μL DCM was added to the reaction. The reaction was stirred at r.t. 15 h 45 m. The reaction was diluted with CH_3CN then co-evaporated with CH_3CN and $PhCH_3$. The residue was triturated with DCM and EtOAc. The crude product was purified further using Sephadex G-15 gel filtration. The coupled product was obtained in 62% yield (8 mg, R_f = 0.34 in 2:1 ^{i}PrOH : 1 M NH_4OAc).

^{1}H-NMR (D_2O – 500 MHz) – δ 5.26 (s, 1 H, Ring **C**-H_1), 5.24 (s, 1 H, Ring **B**-H_1), 4.58 (d, 1 H, J = 8.0 Hz, Ring **A**-H_1), 4.49 (d, 1 H, J = 8.5 Hz, Ring **D**-H_1), 4.44 (d, 1 H, J = 7.0 Hz, Ring **E**-H_1), 4.36 to 4.33 (m, 1 H), 4.27 to 4.21 (m, 3 H), 4.09

(broad singlet, 6 H), 4.01 to 3.59 (m, 62 H), 3.56 to 3.47 (m, 6 H), 1.18 (d, 3 H, J = 6.5 Hz, $H_{6'}$)

^{13}C-NMR (D_2O – 125 MHz) – δ 173.6 & 173.5 (each s', -CH_2-$\underline{C}$ONH-CH_2-), 103.8 (d', Ring **E**-C_1), 103.2 (d', Ring **D**-C_1), 102.4 (d', Ring **A**-C_1), 99.6 (d', Ring **C**-C_1), 94.0 (each d', Ring **B**-C_1), 79.3, 77.1, 76.3, 75.7, 75.6, 75.2, 73.7, 73.6, 73.4, 72.8, 72.0 & 71.9 (each d'), 71.2 & 71.2 (each t'), 70.7 (d'), 70.5, 70.5, 70.5 & 70.4 (each t'), 70.2, 69.4 (d'), 69.3 (t'), 69.0 & 68.7 (each d'), 68.4 (t'), 67.7 & 64.3 (each d'), 62.2, 61.9, 61.0, 40.1 & 39.7 (each t'), 16.2 (q')

LR-ESMS for $C_{48}H_{86}N_2O_{34}Na$ ($M + Na^+$) – Calculated 1257.5; Found 1257.5

Chapter 3

Evaluation of Hetero-divalent Inhibitors*

3.1 Techniques to Evaluate Inhibitors

Isothermal titration calorimetry (ITC), fluorescence and Enzyme-linked immunoassay (ELISA) are some of the techniques that can be used to measure the binding of ligands to their proteins. The common thread for all three techniques is the capability of measuring distinct and minute effects arising from changes in the concentration of the ligand added.

ITC is a thermodynamic technique for monitoring binding when a ligand is introduced to its protein at a constant temperature. Upon interaction of the ligand to the protein, "heat is released or absorbed in direct proportion to the amount of binding".[128] Measurement of this heat allows accurate determination of thermodynamic parameters such as dissociation constants (K_d), reaction stoichiometry (n), enthalpy (ΔH) and entropy (ΔS). From a single experiment a complete thermodynamic profile of the molecular interaction is obtained.

Fluorescence is the measurement of emitted light, most commonly when polyaromatic hydrocarbons or heterocycles are excited. Fluorophores or fluorescent dyes are used as ligands or attached to natural ligands in order to report on the binding to the active site of proteins.[129] Upon addition of the fluorophore to the protein solution, measurement of binding affinity can be deduced from changes in the fluorescence emission spectrum (either wavelength

* All inhibitors evaluations were performed by Dr. Rita Martins in Dr, Hindsgaul's laboratory, Carlsberg Laboratorium in Valby, Denmark, as described on p. 130.

or intensity).[130, 131] An advantage to using fluorescence is the minute amount of protein that is required in order to obtain K_d. Also, the use of fluorophores does not prevent the use of the protein (in subsequent experiments) to examine other ligands.[132]

ELISA rely upon the principles of solid-phase immobilization and the development of colour in order to evaluate the binding between proteins and ligands. Adsorbed ligands are incubated with a solution of the protein that is covalently tagged with an enzyme or can be detected subsequently. When the protein binds to the ligand, the enzyme can catalyze a chemical reaction leading to colour development when a suitable substrate is added.

In all three techniques, minute amounts of proteins and ligands are employed. In the present study, the ELISA technique is used. All three techniques evaluate the binding between a single protein and its ligand, however this study wishes to examine whether simultaneous interactions between two different proteins can occur with a hetero-divalent ligand.

3.2 Binding Affinity vs. Dissociation

The key property of a ligand is its ability to bind effectively to its receptor. The ligand should have specific binding to that receptor or to minor variants of that receptor. This phenomenon is observed throughout biology. However unwanted viruses, bacteria and fungi also make use of such binding to adhere to and penetrate the cellular membrane triggering a cascade of events that

ultimately leads to their replication and survival. From our knowledge of protein-ligand interactions, scientists have attempted to develop molecules that will either bind directly to the virus, bacterium or fungus to prevent infection.

As mentioned earlier, all three techniques (ITC, fluorescence and ELISA) are designed to probe binding affinity (Figure 3.1). Binding affinity and dissociation are opposites in terminology. As displayed in equation 2, the dissociation constant is defined as the individual concentrations of the free protein and free ligand over the concentration of the complex. What is desired in any protein-ligand interaction is great binding affinity (K_a) or low K_d. For use as inhibitory drugs, ligands should have low dissociation constants with a specific receptor and its family. A ligand having a low K_d with its receptor while binding to a great host of other proteins is not what is required. Many lead candidates as drugs have failed due to this type of cross-reaction.

However for this study, the model will be simplified. The purpose of this study is to first to determine whether synthesized ligands will bind to their respective proteins monovalently as well as known monovalent ligands. Then, when covalently linked to **1** via a polyethylene glycol chain are they be able to bind to two different proteins (Con A with anti-IgM antibody and CT and anti-IgM antibody) bind to their ligands simultaneously? If so, how comparable are the dissociation constants to literature?

$$\text{Protein} + \text{Ligand} \underset{k_{OFF}}{\overset{k_{ON}}{\rightleftharpoons}} \text{Protein-Ligand}$$

$$K_a = \frac{[\text{Protein-Ligand}]}{[\text{Protein}] \times [\text{Ligand}]} \qquad \text{(eq. 1)}$$

$$K_d = \frac{[\text{Protein}] \times [\text{Ligand}]}{[\text{Protein-Ligand}]} \qquad \text{(eq. 2)}$$

$$\Delta G = - RT \ln K_a \qquad \text{(eq. 3)}$$

Figure 3.1: Definition of binding affinity and dissociation constants

3.3 Enzyme-linked Immunoassays – Basic Principles

Enzyme-linked Immunoassay (ELISA) is an analytical technique that is based upon antigen-antibody interactions. The ELISA protocol relies upon the practicality of solid-phase immobilization principles along with detection that is both sensitive and selective. Antigens are known to bind tightly to their antibodies and antibodies in general will bind to only one antigen.[133-135] Either the antigen or the antibody is attached to the surface of a microtiter well. For example, antibody will bind to its adsorbed antigen. Detection of this interaction can be performed either by covalently attaching an indicating enzyme (e.g.: horseradish peroxidase, HRP) to the antibody or by adding a second antibody covalently attached to the indicating enzyme; the stem (F_c region) of the primary antibody will behave as the antigen for the secondary antibody. Addition of a substrate (e.g.: *o*-phenylenediamine) to the indicating enzyme will lead to colour

formation when it is oxidized by HRP thereby confirming the primary interaction between the adsorbed antigen and its antibody (Figure 3.2).[132-134]

Experiments where the primary antibody and its antigen have reversed roles can be performed in either a direct (antigen covalently attached to the indicating enzyme) or indirect (the use of a secondary antibody that is covalently attached to the indicating enzyme) fashion. The usefulness of this protocol has expanded the testing of protein-ligand interactions beyond that of antibody-antigen. ELISA also allows for competition studies in order to test new ligands; new ligands can be tested as inhibitors for a toxin or bacterium either directly or indirectly against the known ligand. Quantification of ELISA experiments are measured by taking the concentration of the substrate that results in a 50% inhibition of the antibody binding to the immobilized antigen; this measurement is known as the Inhibition Concentration at 50% (IC_{50}).[132-135]

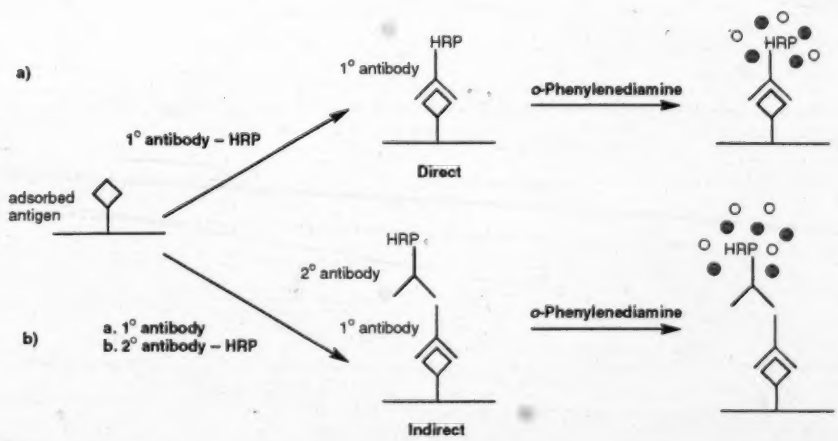


Figure 3.2: ELISA techniques demonstrating a) direct vs. b) indirect conjugation to HRP

3.4 Enzyme-linked Immunoassays – Evaluation Methods

Testing of the synthetic monovalent ligands for Con A and CT (**2** and **3** respectively), will be performed using direct inhibition ELISA studies (Figures 3.3 and 3.4). The lectin and toxin (covalently attached to horseradish peroxidase) are added to microtiter wells on which their respective ligands α-mannopyranoside (**55**) and ganglioside GD_{1b} (**56**), are adsorbed or attached. If **2** and **3** are competitive inhibitors, they will disrupt the interaction between the protein and adsorbed ligand. Upon addition of the substrate (*o*-phenylenediamine), the solution should not colour due to the absence of horseradish peroxidase (Figure 3.3), if the compounds are good inhibitors. Comparison of the concentration of the amount of synthetic ligands added compared to a reference (i.e.: no synthetic ligand addition) should provide an IC_{50} value. For further comparison, commercially available compounds p-nitrophenyl α-D-mannopyranoside (**57**) and lactose (**5**) can also be evaluated for inhibition against their binding proteins (Figure 3.4). α-D-Mannopyranoside (**55**) and ganglioside GD_{1b} (**56**) were used as the adsorbing ligands due to their known binding to their respective proteins.[136-139]

The synthetic hetero-divalent ligands will also be evaluated by inhibition ELISA studies incorporating a multivalent approach. To the microtiter plates containing adsorbed Con A, hetero-divalent ligands **10** or **12** would be introduced individually. Based upon the results from the studies of inhibition using the monovalent ligand **2**, similar levels of binding should occur between both **10** and **12** to Con A, respectively. A solution of monoclonal anti-B antibody would then

be introduced to the exposed terminus of the hetero-divalent ligand (**10** or **12**) featuring the oligosaccharide antigen necessary to bind anti-B antigen antibody. An absorbance reading after incubation with anti-Mouse IgM antibody conjugated to Horseradish Peroxidase followed by the addition of o-phenylenediamine to the microwell would demonstrate the presence or absence of anti-B antibody. Thus, the binding of two different proteins to a hetero-divalent ligand is displayed as a success or a failure (Figure 3.3). The same conditions could be repeated for CT and the synthesized hetero-divalent ligands (**11** and **13**).

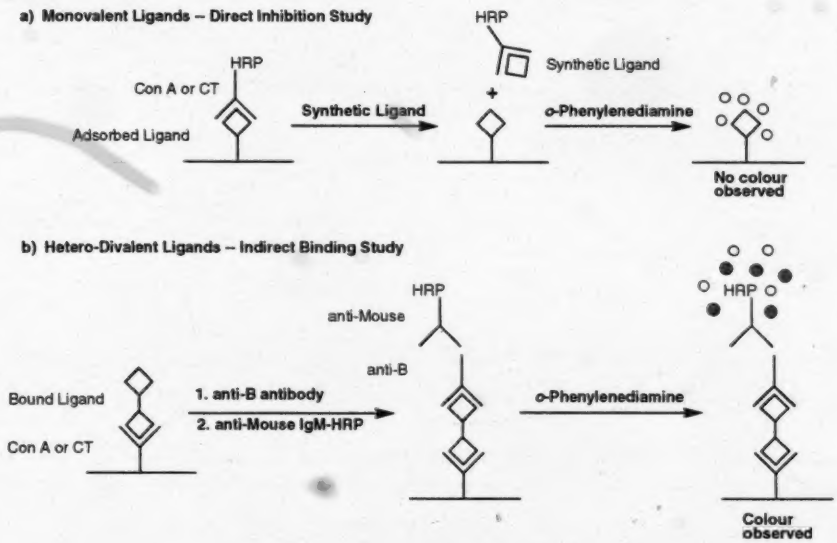


Figure 3.3: Models of ELISA studies: a) monovalent ligands; b) hetero-divalent ligands

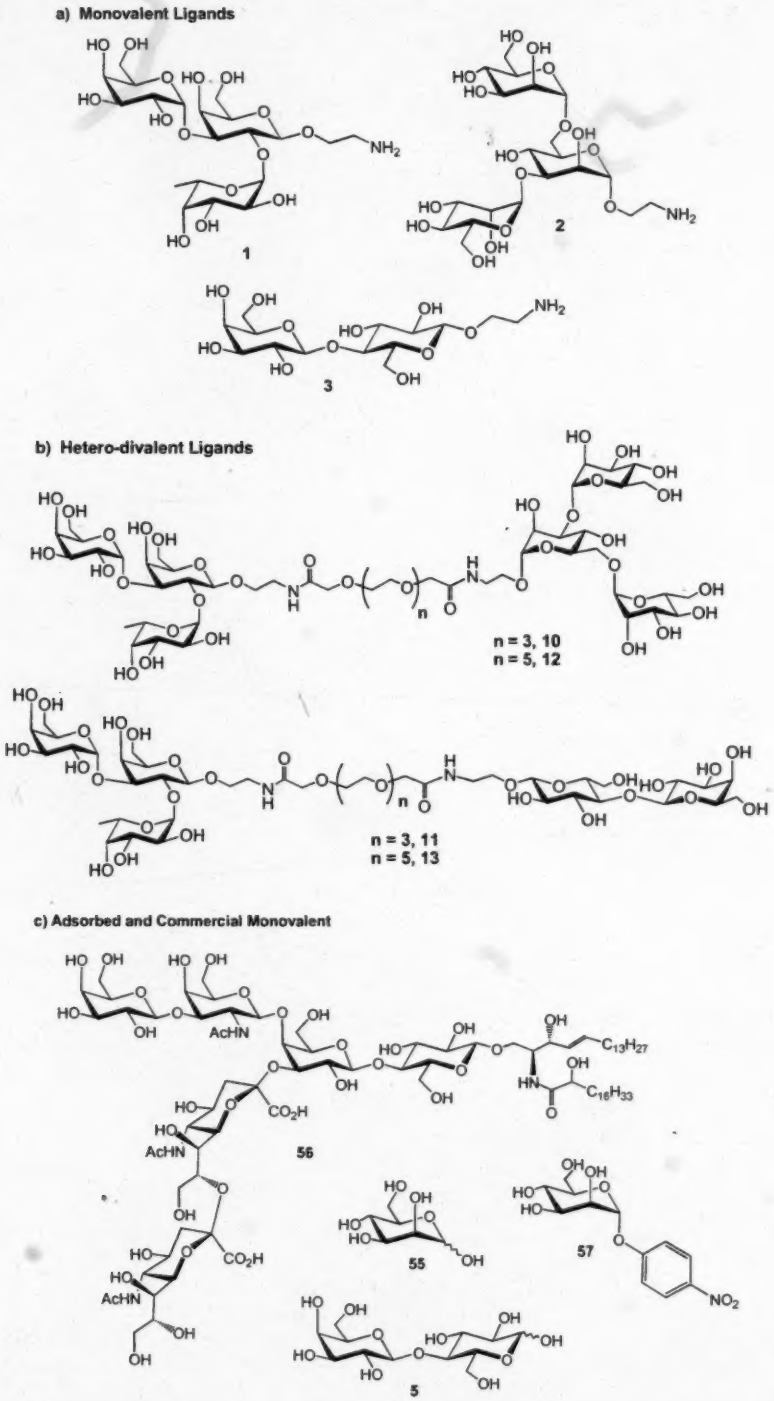


Figure 3.4: Oligosaccharide ligands: a) monovalent; b) hetero-divalent; c) adsorbed and commercial monovalent

3.5 Enzyme-linked Immunoassays -- Results

As mentioned earlier, the inhibitions of Con A and CT were tested using two different procedures (monovalent and multivalent). Both proteins were subjected to evaluations using the mono and hetero-divalent ligands synthesized (Figure 3.3).

The inhibition of Con A was observed when the monovalent ligand **2** was tested. Under these conditions, it was found that the hetero-divalent ligand **10** was also effective at inhibiting the lectin (Figure 3.5). Both of these ligands were compared to a known assay inhibitor of Con A, ligand **57**. The presence of 250 μM **57** (standard assay inhibitor of Con A) gave 58% inhibition of Con A binding to the plate, compared to the 69% and 81% inhibition obtained with only 10 μM of **2** and **10**, respectively (Figure 3.5). These results clearly show that the synthesized compounds are considerably more active as Con A inhibitors. Furthermore of the two compounds, **10** proved to be more potent than **2**. The increase in inhibition may be attributable to the presence of the polyethylene glycol chain in compound **10**.

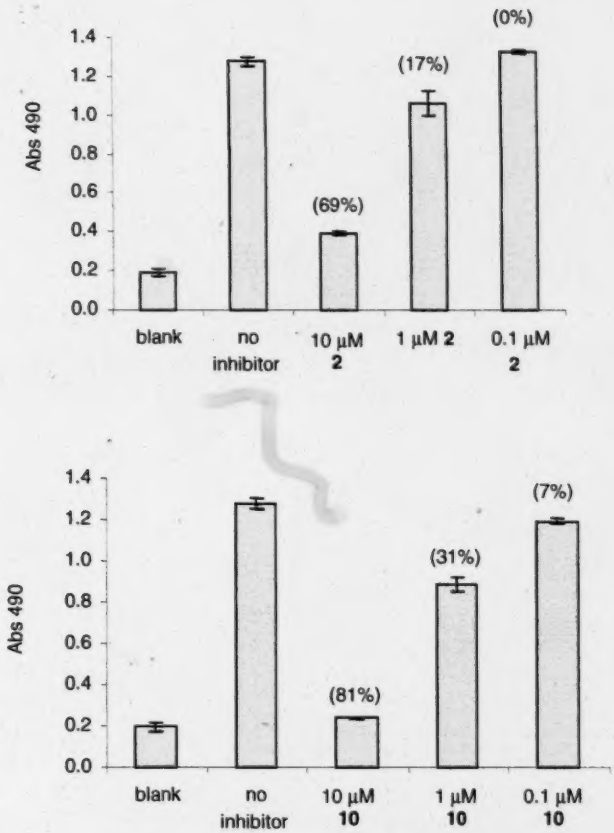


Figure 3.5: Inhibition of Con A binding to plates treated with 55 by the synthesized compounds 2 and 10. bars represent the standard deviations and values in brackets show the relative inhibition (%).

Similar monovalent conditions were applied to CT and ligands **3** and **5**. Once again, a much smaller concentration of the synthesized ligand **3** was used, yet resulting in approximately 10 times higher potency than **5** as an inhibitor for CT binding; the inhibitory effect was 29% for 10 mM **5** compared to 36% for only 1 mM of compound **3** (Figure 3.6).

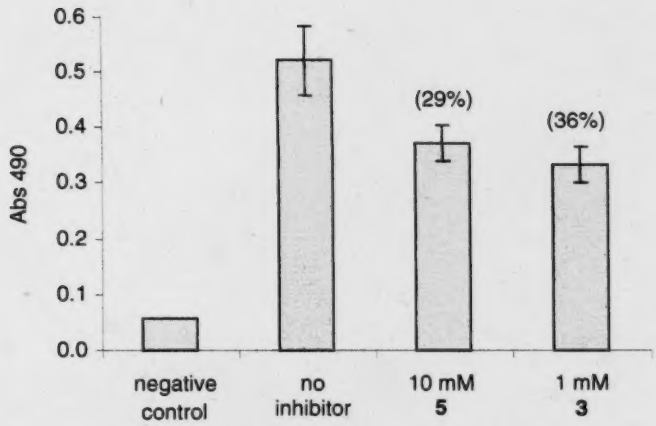


Figure 3.6: Inhibition of CT binding to plates treated with 56 by 5 and 3. bars represent the standard deviations and values in brackets show the relative inhibition (%).

Assays, under multivalent conditions gave very disappointing results. The ligand **10** was unsuccessful at binding both Con A and monoclonal anti-B antibody simultaneously. A similar result was obtained when ligand **11** was tested against CT and the monoclonal anti-B antibody.

A number of reasons may explain these negative results. Molecular modeling studies yielded lengths approximately 40 Å for the ligands **10** and **11**. This distance may not have been long enough to accommodate both proteins to bind simultaneously (Figure 3.7); the lengths were based upon the presumption that the polyethylene glycol chain was adopting anti-periplanar geometry. Geometry of the proteins also plays a factor in the binding. In order for binding to occur, the quarternary structures of the proteins should not be distorted, thereby facilitating the binding of proteins (Con A to monoclonal anti-B antibody and CT to monoclonal anti-B antibody) to their respective binding oligosaccharides at the

termini of the ligands. Thus the measurement of the ligands using molecular modeling may not have accurately represented the true conformation of the polyethylene glycol portion of the ligands in solution.

Another factor to take into consideration is the entropy for the monoclonal anti-B antibody to bind to its oligosaccharide ligands (**10** and **11**) in the presence of both Con A and CT was too high. In solution, the polyethylene glycol chain may be in constant conformational flux, thereby limiting the opportunities for the monoclonal anti-B antibody to be able to bind to its oligosaccharide antigen. Even if there was sufficient length and the entropy was small enough to permit docking, steric interactions between the two proteins (Con A to monoclonal anti-B antibody and CT to monoclonal anti-B antibody) may also have prevented the monoclonal anti-B antibody from properly accommodating its oligosaccharide antigen into the binding site. Though there are to date no crystal structures of the monoclonal anti-B antibody in order to properly evaluate this explanation, it is reasonable that amino acid residues residing on the periphery of the proteins may have prevented proper docking of the monoclonal anti-B antibody to its oligosaccharide antigen. What is also not known is the depth of the binding pocket for the monoclonal anti-B antibody. It is possible that the overall lengths of **10** and **11** may not have permitted their antigen portions to be properly incorporated into the binding site of the monoclonal anti-B antibody avoiding steric interactions between the proteins.

An additional impediment was restricted access to CT derivatives (up to a one year delay) due to bioterrorism regulations. Based upon the results from the

testing of ligands **10** and **11** along with governmental restrictions, ligands **12** and **13** were not tested.

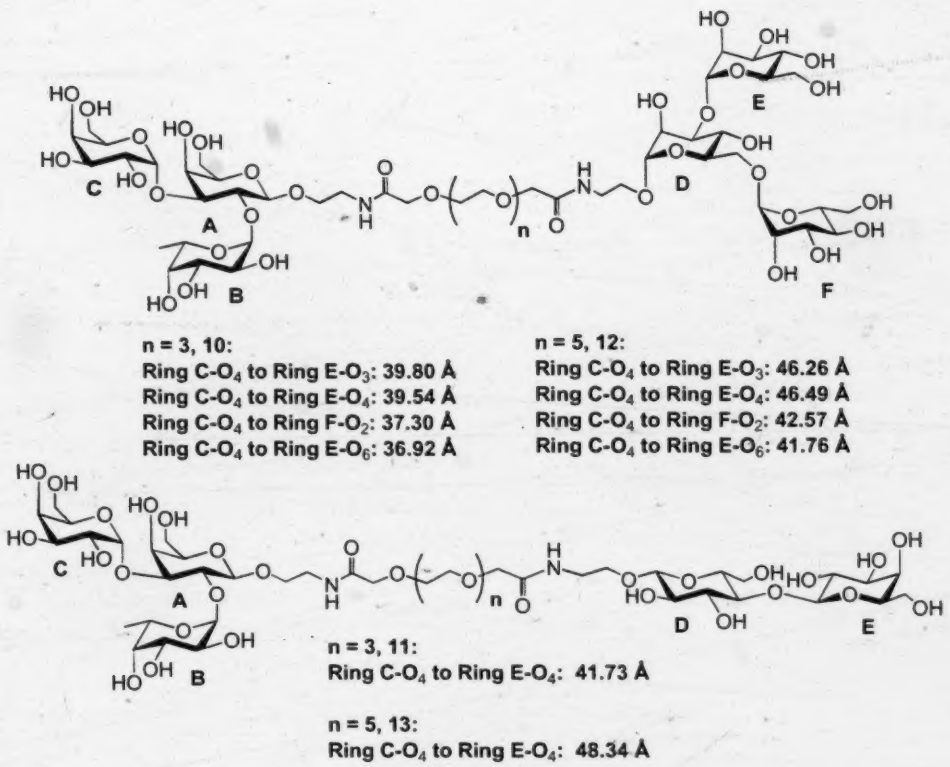

n = 3, 10:
Ring C-O4 to Ring E-O3: 39.80 Å
Ring C-O4 to Ring E-O4: 39.54 Å
Ring C-O4 to Ring F-O2: 37.30 Å
Ring C-O4 to Ring E-O6: 36.92 Å
n = 5, 12:
Ring C-O4 to Ring E-O3: 46.26 Å
Ring C-O4 to Ring E-O4: 46.49 Å
Ring C-O4 to Ring F-O2: 42.57 Å
Ring C-O4 to Ring E-O6: 41.76 Å
n = 3, 11:
Ring C-O4 to Ring E-O4: 41.73 Å
n = 5, 13:
Ring C-O4 to Ring E-O4: 48.34 Å

Figure 3.7: Lengths of hetero-divalent ligands

3.6 Experimental

General Methods and Materials

All experiments were performed by Dr. Rita Martins in Dr. Hindsgaul's laboratory at the Carlsberg Laboratorium in Valby, Denmark. The experiments modified (from references cited) are described below.

Biological and chemical reagents were obtained from the following sources:

For monovalency studies:

Disialoganglioside from bovine brain (GD_{1b}, G8146), 4-Nitrophenyl α-D-mannopyranoside (N2127), *o*-Phenylenediamine dihydrochloride (OPD, P1526), Bovine serum albumin (BSA, A4503), and Concanavalin A peroxidase-conjugate (Concanavalin A-HRP, L6397) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cholera toxin peroxidase-conjugate (CT-HRP, #105) was obtained from List Biological Laboratories, Inc. (Canada). α-D-mannopyranoside (SW-01-009) and N-Acetyl (blank, SW-00-001) GlycoWell™ 96-well microtiter plates were obtained from Lundonia Biotech (Lund, Sweden).

For multivalency studies:

Concanavalin A Type IV (Concanavalin A, C2010), Cholera toxin B subunit (C9903), and Peroxidase-conjugate anti-mouse IgM antibody (A8786) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Monoclonal anti-B antigen antibody (mouse IgM) was from Abcam (Cambridge, UK).

Polystyrene 96-well microtiter plates (Kartell Art. no. 02620-00) were purchased from VWR (Sweden). Lactose was obtained from Carlsberg Laboratorium (Valby, Denmark). All other reagents were of analytical grade.

Phosphate buffered saline (PBS, pH 7.2) was a 10 mM potassium phosphate buffer, pH 7.2, containing 150 mM NaCl. PBS-Tween 20 (0.05%) was prepared from the addition of 0.10 mL of Tween 20 into 200 mL PBS. BSA-PBS (0.1%) was prepared from the addition of 0.1 g of BSA in 100 mL PBS. Cova

buffer consisted of a 2 M NaCl, 40 mM Mg_2SO_4-$7H_2O$ and 0.05% Tween 20 solution. Citrate-phosphate buffer (pH 5.0) was composed of a 34 mM citric acid and 66 mM sodium phosphate. *o*-Phenylenediamine (OPD) solution, i.e.: substrate solution, was prepared from the addition of 1 mg *o*-phenylenediamine dihydrochloride per mL 100 mM Citrate-phosphate buffer (pH 5.0). Optical densities were read using an ELISA plate reader (DYNEX Technologies, MRX TC Revelation).

Molecular modeling experiments were performed at the University of Alberta using Biosim Molecular Modeling Package with Insight II and Discover Modules. All experiments were performed on the SGI Indigo II.

Experimental Procedure for Cholera toxin (G_{D1b} Enzyme-linked adhesion assay)

G_{D1b} solution (0.5 μg/ml) in PBS (100 μl) was placed in the wells of a plastic plate and kept overnight at 4 °C. The plate was then washed three times with PBS solution. To each well was added 200 μl of BSA-PBS solution. The plate was incubated for 1 h at room temperature and washed three times with PBS solution. Sample mixtures containing CT-HRP (0.4 μg/ml) and inhibitor (**3** or **5**) in 0.1% BSA-PBS were incubated for 1 h at room temperature prior to their addition (100 μl) to the wells and further incubation for 30 min at room temperature. After washing three times with PBS solution, 100 μl of the substrate solution (pH 5.0) was added to the wells, the plate was incubated at room

temperature, and Abs_{490} was read after 30 min. Negative controls were performed by the same experiments on wells not coated with G_{D1b}.

Experimental Procedure for IgM-HRP using Bound Cholera Toxin

Plates were coated with Cholera toxin (100 μl; 10 μg/ml in PBS, overnight at 4 °C), washed twice with PBS solution, followed by coating with 1% BSA-PBS (200 μl, 1 h at r.t.), and washed three times with PBS solution. Compound **11** (50 μl; 200 μM in 0.1% BSA-PBS) was added to the wells and incubated for 1 h hour at r.t. Monoclonal anti-B antigen antibody (50 μl; 10/25/100 times diluted in 0.1% BSA-PBS) was added and allowed to form multivalent complexes with **11** for 2 h at room temperature. The solution mixture was removed from the well and peroxidase-conjugate anti-mouse IgM antibody (100 μl; 0.5 μg/ml in 0.1% BSA-PBS) was added and incubated for 2 h at room temperature. After washing three times with PBS, 100 μl of OPD solution was added to the wells, the plate was incubated at room temperature, and Abs_{490} was read after 30 min.

Experimental Procedure for Concanavalin A-Horseradish Peroxidase

Inhibitor solution (compounds **2, 10** or **57**; 50 μl) in PBS-Tween 20 was added to the wells of a α-D-mannopyranoside (**55**) plate, followed by the addition of 40 μg/ml Con A-HRP solution (50 μl) in PBS-Tween 20. The plate was incubated for 45 min at room temperature and washed three times with Cova

buffer and once with 100 mM citrate-phosphate buffer (pH 5.0). Substrate solution (100 µl) was added to the wells, the plate was incubated at room temperature, and Abs_{490} was read after 30 min. Negative controls were set by performing the same experiments on N-Acetyl plates, where no carbohydrate moiety is attached.

Experimental Procedure for IgM-HRP using Bound Concanavalin A

Plates were coated with Con A (100 µl; 1 mg/ml in PBS, overnight at 4 °C), washed three times with PBS-Tween 20, followed by coating with BSA (200 µl; 1% in PBS-Tween 20, 1 h at room temperature) and washed three times with PBS-Tween 20. Compound **10** (50 µl; 80/200 µM in PBS-Tween 20) was added to the wells and incubated for 1 h at room temperature. Monoclonal anti-B antigen antibody (50 µl; 10/25 times diluted in PBS-Tween 20) was added and allowed to form multivalent complexes with **10** for 2 h at room temperature. The solution mixture was removed from the well and Peroxidase-conjugate anti-mouse IgM antibody (100 µl; 0,8 µg/ml in PBS-Tween 20) was added and incubated for 2 h at room temperature. After washing three times with PBS-Tween 20, 100 µl of the substrate solution (pH 5.0) was added to the wells, the plate was incubated at room temperature, and Abs_{490} was read after 30 min.

Chapter 4

Future Work

Though the study failed to materialize the desired results, there were some positive indications that hetero-divalent ligands may perhaps bind different proteins simultaneously. Using direct inhibition studies, monovalent ligands **2** and **3** were successful at competitively inhibiting their respective proteins. However, monovalent ligand **1** was not tested under these conditions to measure its inhibitory capabilities.

It was also demonstrated that hetero-divalent ligand **10** was a more potent inhibitor against Con A than its monovalent version (**2**). Given that polyethylene glycols have favourable biocompatible properties, it would be worthwhile to examine whether hetero-divalent ligand **12** would provide equal inhibitory capability compared to **10** against Con A. When measuring inhibition potency against CT, neither of the hetero-divalent ligands **11** and **13** were tested. Performing these tests, would provide greater strength in the use of polyethylene glycols as suitable linkers.

Though in-direct binding studies of hetero-divalent ligands **10** and **11** were found to be unsuccessful, ligands **12** and **13** may prove the hypothesis correct. The additional length of approximately 10 Å may provide sufficient space to reduce steric hinderances between the amino acid residues at the periphery of the proteins. This added length may also facilitate the binding of the ligands without distorting the quarternary geometry of the proteins.

If ligand **1** is successful at inhibiting monoclonal anti-B antibody (thereby confirming its structure), however hetero-divalent ligands **12** and **13** are still unsuccessful at binding two different proteins simultaneously then a revised approach is needed. Though longer polyethylene glycol chains may prove to be useful, overcoming entropy will play a larger role in determining whether binding will occur.

The use of dendrimers may provide the necessary solution. Growth of large dendrimers is highly plausible. There has been enough development in the field to facilitate the synthesis of a large dendrimer that can present the human blood group b determinant (**1**) on one half of the periphery and either of the other ligands (**2** or **3**) on the other side of the periphery of dendrimer. This type of dendrimer may well provide ample space between the two proteins while the more rigid structure of dendrimers may reduce the entropy for successful binding.

Chapter 5

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